Monograph No. 22

Evaluation of Chemicals for Oculotoxicity

November 1994

ISSN-0773-6347-22

Brussels, November 1994

ECETOC Monograph No. 22

EVALUATION OF CHEMICALS FOR OCULOTOXICITY

CONTENTS

SUMMARY

Reports on the outbreak of ocular disease purported to be associated with long term exposure to chemicals, in particular to some widely used organophosphorus agents, have raised legitimate concern about the sensitivity and reliability of routine toxicity testing for the detection of ocular effects. This has prompted at least one regulatory agency to request that special techniques of examination should be used to supplement existing testing methods. In response to this situation ECETOC has reviewed the available evidence on the assessment of oculotoxicity and this report recommends a practical testing strategy for evaluating the oculotoxic potential of chemicals.

The major target sites within the eye have been described together with the methods of examination. Examples of oculotoxic effects in man, especially those produced with agrochemicals and industrial chemicals have been compared with corresponding animal studies. Whilst some of the specialised animal models designed to replicate human ocular disease provide an insight into its pathogenesis, the correlation between oculotoxicity in man and animal safety evaluation studies is generally poor, since animal studies may show effects irrelevant for human beings.

Retrospective analysis of the data shows that the causal relationship between exposure to some chemicals and impairment of the visual system in man is possible, and that many of these adverse effects can be detected and reproduced in animal studies. To achieve this, routine toxicology testing should be performed in a stepwise manner and supplemented by additional technical procedures when oculotoxicity is anticipated to occur. Most chemically-induced changes are detected using a combination of ophthalmology and histopathology; other more specialised techniques are only needed rarely. The existing toxicity guidelines in animals cover in principle all testing requirements, although their harmonisation by regulatory agencies internationally would be of benefit to both industry and the toxicology community.

It is recommended, that in addition to the requirements of repeat dose toxicity studies, histopathology of the eye should be incorporated into those studies where the design includes histopathological examination of the nervous system, as a means of assessing the neurotoxic potential of a chemical. Furthermore, histopathology of the ocular adrexa such as the lacrimal and Harderian glands may be essential for the interpretation of some findings in the eye.

In conclusion, a practical testing strategy for oculotoxicity which utilises the framework of existing guidelines but which equally retains the flexibility to extend the range of technical procedures to

address specific oculotoxic effects is considered to offer the best approach to hazard identification and hence to prevention of toxicity in man.

SECTION 1. INTRODUCTION

Vision is one of the most essential human functions. It therefore follows that there is concern about potential adverse effects caused by chemicals on the visual system. Chemicals used for medical treatment or applied as cosmetics to the eye and surrounding areas are carefully tested for efficacy and safety. The possibility of systemic effects of such chemicals applied locally to the ocular area must also be taken into consideration. Conversely possible ocular effects of chemicals applied by other routes must also be identified and assessed in safety tests. Additionally the potential adverse ocular effects of agricultural chemicals which find wide dispersive application into the environment and which may inadvertently reach the human body must be avoided as far as possible.

Recognising the importance of these issues a Task Force was established with the following Terms of Reference:

- evaluate the evidence for ocular toxicity in man following long-term exposure to chemical substances;
- review the relevance of existing guidelines and test methods for the evaluation of oculotoxic potential of substances;
- recommend a practical strategy, including details of test methods, to be employed for the evaluation of oculotoxic potential of chemical substances.

Substantial progress has been made in ocular toxicology with respect to the safety of medicaments and cosmetics which is reflected in specific test methods and manuals covering a wide spectrum of experimental and clinical aspects (Chiou, 1992; Hockwin *et al*, 1992b). Earlier, special attention has been devoted to testing for eye irritation (ECETOC, 1988; Workshop on Updating Eye Irritation Test Methods, 1993) and consequently this topic is excluded from evaluation in this review.

Although this review will consider all categories of chemical agents with oculotoxic potential it is mainly focused on pesticides and industrial chemicals. For example, a possible association between human exposure to organophosphate insecticides and ocular toxicity was suggested (Ishikawa, 1971, 1973, 1978) and the question has been raised whether these effects can be reproduced in laboratory animal experiments (Boyes *et al*, 1994). Since knowledge of acute and subchronic effects is a prerequisite for understanding chronic toxicity of particular chemicals, this review considers acute effects along with the effects of long-term exposure.

The goal of this report is to provide guidance for the safe and efficient conduct of toxicological testing based on available scientific evidence and the practical experience of toxicologists actively working in the field. The first section defines the potential target sites in the visual system, explains their functional anatomy and specific susceptibility. Subsequently the evidence for ocular toxicity in man is reviewed and the merits and shortcomings of the available animal models are commented upon. Further sections present an overview of diagnostic methods for ocular toxicity, analyse and compare the current testing guidelines and propose a strategy for optimal testing. A glossary of terms is provided for the assistance of the non-specialised reader.

SECTION 2. SPECIFIC OCULAR TISSUES AS TARGETS OF TOXICITY

2.1 INTRODUCTION

Ocular toxicity comprises not only the toxic effects on the eye and the optic nerve, but also those affecting the adnexa i.e the eyelids, lacrimal glands, or extraocular muscles. Consideration of the tissues anatomically and functionally associated with the eye as a target of ocular toxicity is essential for understanding the mechanisms of any effects observed, so that the appropriate interpretation in terms of causal relationships can be made. For instance, corneal and conjunctival lesions may be caused by a failure of sensory innervation, or by lacrimal malfunction, rather than representing a primary effect on the eye.

The essential parts of the eye are demonstrated in Figure 1, and their anatomical and functional features described in the context of characteristic pathological reactions, relevant species differences, species-specific spontaneous lesions, and particular susceptibility to intoxication. Most of the data quoted in the following sections originate from Millichamp's contributions in "Ophthalmic Toxicology" (1992a,b) which should be referred to for more detail.

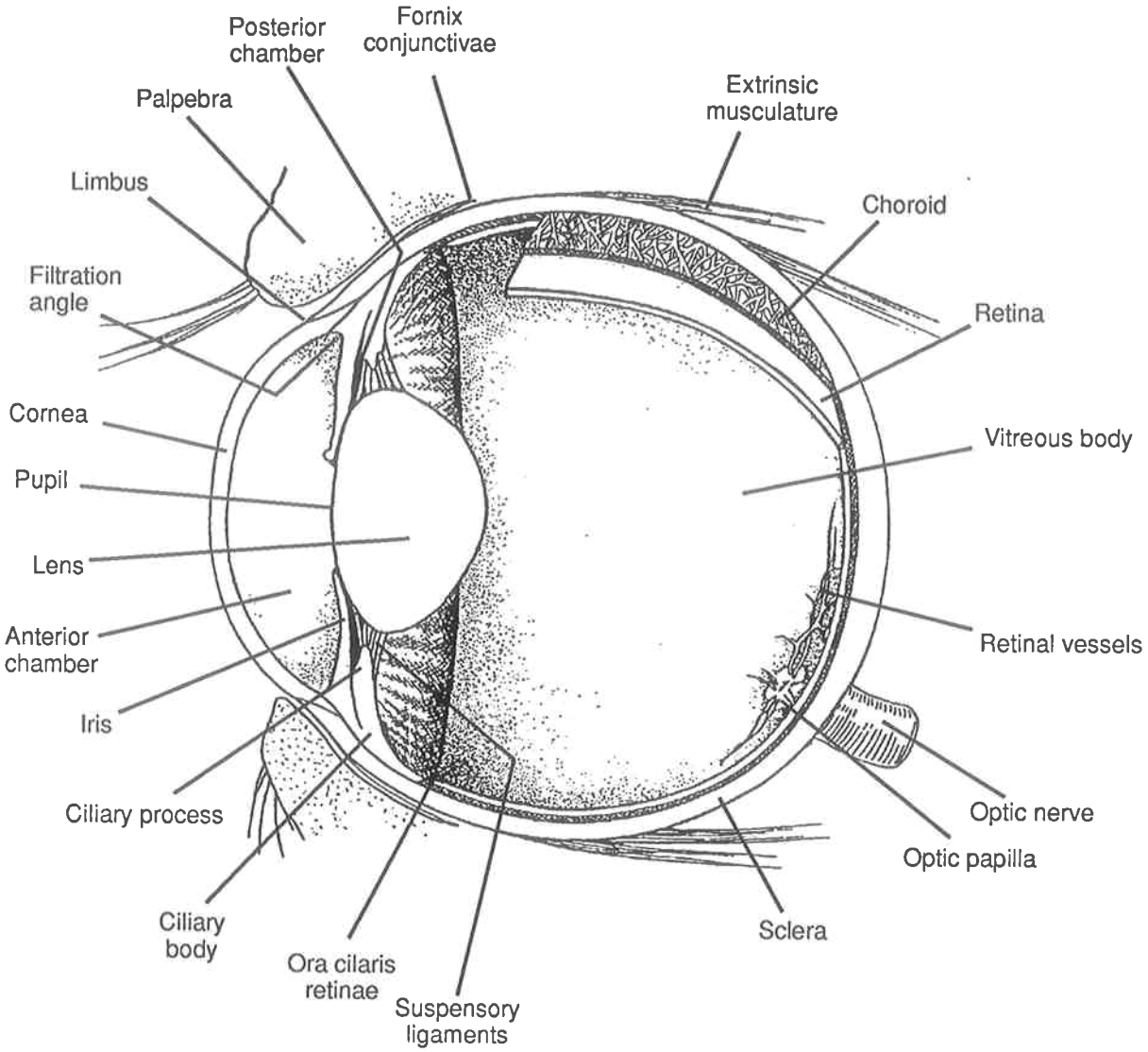
2.2 ANATOMICAL FEATURES

2.2.1 Conjunctiva and Cornea

The conjunctiva is a mucous membrane covering the inner surface of the eyelids (palpebral conjunctiva) and the anterior part of the sclera (bulbar conjunctiva). The transition area of both parts is called the conjunctival fornix and the space delineated by the conjunctival and corneal surface is the conjunctival sac. The conjunctiva is composed of a superficial layer of non-keratinising stratified squamous epithelium and an underlying substantia propria. The palpebral conjunctiva contains goblet cells which are particularly numerous in the area of the fornix.

The cornea forms the anterior transparent part of the outer coat of the eye, the remaining part of which is formed by the sclera. The cornea consists of five layers: non-keratinising stratified squamous epithelium, Bowman's membrane, collagenous stroma, Descemet's membrane, and an endothelial layer. In most domestic and experimental animals, with the exception of non-human primates, Bowman's membrane is indistinct even by electron microscopy.

Figure 1 Gross Anatomy of the Eye (Vertical Section)



(reproduced from: Applied Veterinary Histology, William J. Banks, 1981, Williams and Wilkins, Baltimore/London)

The blood supply of the conjunctiva is derived from the anterior ciliary arteries and the vascular arcades of the eyelids. The conjunctiva and cornea are innervated by the trigeminal nerve.

2.2.2 Anterior Uvea

The uvea forms the "middle coat" of the eye and is composed of the anterior part, comprising the iris and the ciliary body, and the posterior part, comprising the choroid and, in tapetal species, the tapetum lucidum.

The iris possesses both dilator and constrictor muscles, which regulate the size of the pupil. The stroma of iris contains collagen fibres, melanocytes (devoid of pigment in albinos) and fibroblasts. The posterior surface of the iris is covered by epithelium which is pigmented in pigmented individuals. The ciliary body supports the lens and enables accommodation by changing lens shape using the ciliary muscle. The ability to accommodate varies from species to species being more extensive in primates, dogs and rabbits, and less extensive in rodents. Ciliary processes project from the ciliary body towards the lens and consist of highly vascular collagenous stroma covered by ciliary epithelium, responsible for secretion of aqueous humour. The iridocorneal angle, located anterior to the ciliary body, between the iris and the cornea, is the primary drainage site of aqueous humour from the eye.

2.2.3 Posterior Uvea

The choroid forms the "middle coat" of the posterior eye and is located between the sclera and the retina. It is separated from the retina by Bruch's membrane. The choroid consists mainly of blood vessels, collagen, fibroblasts and melanocytes. Unlike retinal capillaries, the choroidal capillaries are fenestrated and therefore devoid of barrier function. In several animal species the choroid is modified by the presence of the tapetum lucidum, which is cellular in carnivores and fibrous in herbivores.

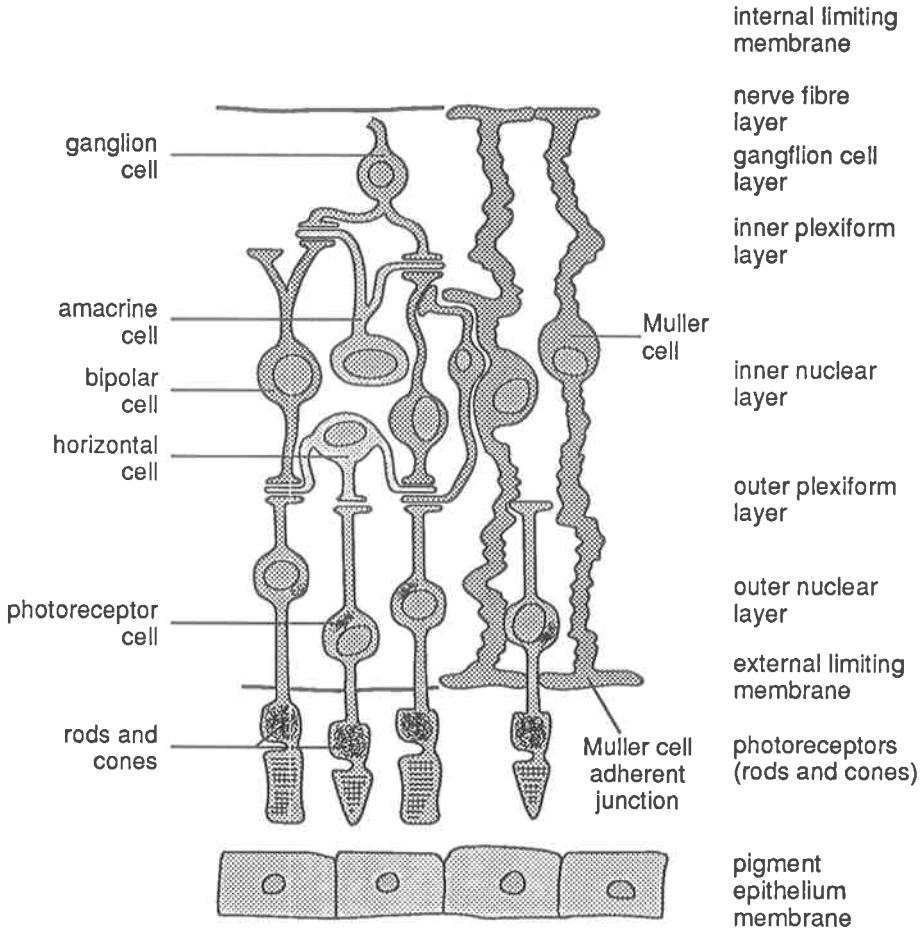
2.2.4 Lens

The lens of the eye is an epithelial structure, composed of an inner nucleus, surrounded by a cortex and enclosed by a capsule. The nucleus and cortex consist of lens fibres, which are continuously formed by a single layer of epithelial cells located under the anterior lens capsule. The anterior capsule overlying the epithelial cells is much thicker than the posterior. Geographical terms "equator" and "poles" are used to describe topographical areas of the lens: the equator pertains to the peripheral margin between anterior and posterior surfaces, whilst the poles are the central regions of the anterior and posterior surfaces.

2.2.5 Retina (See Figure 2)

The retina lines the inner surface of the posterior segment of the eye and consists of an inner layer called the neural retina and an outer layer called the retinal pigment epithelium. The retinal pigment epithelium is a single layer of cuboidal cells lying on Bruch's membrane of the choroid, and contains variable amounts of melanin pigment (none in albinos). The inner surface of each cell forms processes extending between the outer segments of the photoreceptors. The retinal pigment

Figure 2 Diagrammatic Representation of the Mammalian Retina



(reproduced from: Histology, Alan Stevens and James Lowe, 1992, Gower Medical Publishing, London/New York)

epithelial cells internalise shed photoreceptor outer segments. In addition to this "waste removal" the retinal pigment epithelium provides nutrition to the outer layers of the retina and forms the outer part of the blood-retinal barrier. The photoreceptors form the outermost layer of the neural retina and include the rods and cones. Their outer segments consist of stacks of membrane-bound discs, while the inner segments are highly metabolically active, containing numerous mitochondria. The cell bodies of the photoreceptor cells with their nuclei form the outer nuclear layer and their axons synapse with bipolar and horizontal cells in the outer plexiform layer. Nuclei of the bipolar, horizontal, amacrine, and retinal glial (Müller) cells are located in the inner nuclear layer. Cell processes form the inner plexiform layer lying between the inner nuclear and the ganglion cell layer. The ganglion cells send their processes into the nerve fibre layer, the fibres of which converge towards the optic nerve papilla and form the optic nerve. Unlike most other species, in the rabbit these fibres are already myelinated before they form the optic nerve, so that toxic damage to myelin

may be assessed in the rabbit eye. Flat expansions of the Müller cells at the surface of the vitreous body represent the internal limiting membrane which separates the retina from the vitreous body.

The proportion and distribution of the rod and cone photoreceptors varies considerably between species. In species with high visual acuity the retina is rich in cones, which may be especially concentrated in one region (macula lutea, area centralis), or may be evenly distributed through the whole retina. Primates possess the macula lutea with a fovea centralis, which is devoid of blood vessels and rod photoreceptors and contains an especially high cone density. The corresponding structure in other species is the central area which may be round or a narrow horizontal area, the so called "horizontal streak". In Beagle dogs the form of the central area shows considerable inter-individual variability and contains features of both the round central area and the horizontal streak (Krinke *et al*, 1981). Nocturnal species such as rodents possess a retina composed predominantly of rods, and are devoid of retinal centralisation.

The blood supply to the retina comes from two sources. The choroidal circulation provides oxygen and nutrients to the outer part of the retina, including the photoreceptors. The retinal pigment epithelium controls the flow of nutrients and metabolites into and out of the neural retina. The other source of the blood supply is the retinal vessels; in primates there is a central retinal artery, and in other species with retinal vasculature there are posterior ciliary arteries forming cilioretinal arteries. In primates the whole eye is supplied by the internal carotid artery via the internal ophthalmic artery. In domestic animals the optic nerve is also supplied from the internal ophthalmic artery, whereas the choroid and retina is supplied from the external maxillary artery via the external ophthalmic artery. A particular feature of the bird eye is the presence of a highly vascular organ called the pecten.

2.2.6 Optic Nerve

The optic nerve connects the eye to the brain. The extracerebral part of the nerve is known as the optic fascicle, the intracerebral part forms the optic tract. The left and right nerves cross and partially exchange their fibres in the optic chiasm: in the rat about 90%, in the dog about 75% and in man about 50% of fibres cross to the opposite side. Histologically the optic nerve belongs to the central nervous system, i.e. the axons of ganglionic cells are invested with myelin sheaths formed by oligodendroglial cells and surrounded by astrocytes and connective tissue sheaths continuous with the cerebral meninges. The endothelial cells of the blood vessels also have "tight junctions" providing a blood-tissue barrier as in the central nervous system except at the lamina cribosa. The site in the sclera where the nerve fibres leave the eye is the lamina cribrosa, a sieve-like arrangement of scleral collagen through which fascicles of nerve fibres pass. Unlike the peripheral

nerves, the optic nerve is unable to regenerate following injury resulting in disruption of its nerve fibres.

2.2.7 Eyelids

The eyelids consist of outer skin and muscles and inner connective tissue lined by the palpebral conjunctiva. In primates the connective tissue layer forms the tarsal plate in the upper lid, to which muscles elevating the lid are attached. In other species there is a poorly developed perimarginal tarsal plate. Tarsal, or Meibomian glands located in the upper and lower lid are modified sebaceous glands contributing lipid to the tear film. The lid muscles include the orbicularis muscle which closes the lids, the levator palpebrae superioris and the sympathetically innervated musculus tarsalis superior (Müller's muscle). Numerous animal species, but not rodents, possess a third eyelid (nictitating membrane).

2.2.8 Lacrimal System

The tear film provides wetting, lubrication, nutrition, non-specific and specific defence against infection, and removes waste products. The tear film is produced by the major ocular glands including the lacrimal, the Harderian, and the gland of the nictitating membrane, and the minor ocular glands, such as the Meibomian glands and the conjunctival goblet cells. The major ocular glands are not fully represented in all species: in man and other primates only the lacrimal gland is present; in dogs and cats the lacrimal and the nictitans gland occur; in rodents there are lacrimal and Harderian glands, and in rabbits lacrimal, Harderian and nictitans glands are found. The Harderian gland is tubuloalveolar, devoid of an intraglandular duct system, and produces secretory material containing lipid, porphyrin and melatonin. The lipid-containing secretion may serve to lubricate the eye, i.e. it can compensate for breakdown of the tear film. The gland is also considered to serve as an extraretinal photoreceptor, to have a pheromonal function and, owing to the porphyrin content, a photoprotective function. The lacrimal gland is a serous, tubuloacinar gland with numerous excretory ducts. Secretory activity of both Harderian and lacrimal glands is stimulated by parasympathicomimetic compounds. Excessive porphyrin release from the Harderian gland in rodents is responsible for a "blood-caked" appearance, or chromodacryorrhoea.

2.2.9 Extraocular Muscles

In species capable of ocular movement, the extraocular muscles include the rectus, oblique and retractor muscles, innervated by the oculomotor, trochlear and abducens nerves.

2.3 PATHOLOGICAL REACTIONS

2.3.1 Conjunctiva and Cornea

Inflammatory conditions of the conjunctiva are called conjunctivitis, those of the cornea, keratitis. Follicular conjunctivitis pertains to inflammations associated with hyperplasia of conjunctiva-associated lymphoid tissue. Pathological lesions consisting of loss of tissue include erosions of the corneal epithelium, progressing to corneal ulcers when the basement membrane of stratified squamous epithelium is damaged, or perforation of the cornea following damage to Descemet's membrane. Neovascularization of the corneal stroma results from production of granulation tissue after severe stromal injury. Additional corneal reactions include: stromal oedema, corneal deposits, lipidosis and mucopolysaccharidosis.

2.3.2 Anterior Uvea

A characteristic pathological reaction of the uvea is an irritative response and uveitis. The ocular irritation consists of an anterior uveal hyperaemia, miosis, breakdown of the blood-aqueous barrier and a change (usually a rise) in intraocular pressure. Uveitis is associated with cellular infiltration into the anterior segment of the eye. The rabbit is the most sensitive species to ocular irritation. The initial irritation is mediated by a neural reflex arc releasing chemical mediators (substance P, calcitonin gene-related peptide) from trigeminal nerve endings in the anterior uvea in response to sensory stimulation of the cornea and conjunctiva. The sensitivity of the blood-aqueous barrier to breakdown is higher in rabbits than in other species, and may be associated with the presence of well developed ciliary processes in the rabbit. Miosis results from contraction of the iris sphincter muscle, which consists of smooth muscle fibres containing muscarinic cholinergic receptors in mammals and nicotinic cholinergic receptors in lower vertebrates such as birds and reptiles. Further reactions include inflammatory changes of the iris, the formation of iridial cysts and elevated intraocular pressure.

2.3.3 Posterior Uvea

Characteristic pathological changes of the posterior uvea are inflammation (choroiditis), oedema, necrosis, and tapetal colour change.

2.3.4 Lens

The most common and significant abnormality of the lens is cataract. It is defined as "opacity", or "loss of transparency" of the lens. The formation of cataracts is associated with changes in lens

proteins, increase in insoluble protein, glycosylation and formation of high molecular weight aggregates. Differences in the lens metabolism of different species may account for specific sensitivities to the development of toxic cataract. A notable example of such a species difference is the cataractogenicity of 2,4-dinitrophenol in chickens. This agent was designed as a slimming drug and when tested in rabbits and dogs, did not produce cataracts. In man, however, it produced reversible lens opacities. Later investigations showed a high susceptibility of the chicken lens to this agent, which uncouples oxidative phosphorylation and decreases the formation of ATP, which may be more important for preservation of the intact structure of the chicken as opposed to the rabbit or canine lens (Hockwin *et al*, 1992a).

Differential diagnosis of cataract must take into consideration the normal anatomical features of the lens, e.g. the Y-shaped "suture lines" in dogs, cats and man, which must not be confused with cataracts. Further reactions include lens deposits, lamellated inclusions, water clefts and vacuoles.

2.3.5 Retina

Characteristic pathological reactions of the retina include oedema, detachment, haemorrhage, vasoconstriction or vasodilation, retinal lipidosis and changes in pigmentation, degeneration of the pigment epithelium, photoreceptors, retinal neurones or glia resulting in atrophy.

2.3.6 Optic Nerve

Pathological conditions of the optic nerve include axonal degeneration, myelin oedema and demyelination.

2.3.7 Eyelids

Pathological conditions include entropion (roll-lid), ectropion (turning outward of the eyelid), ptosis (drooping of the upper lid) and blepharitis (lid inflammation). Blepharospasm (spasm causing closure of the eyelids), is a common reaction to a painful sensation.

2.3.8 Lacrimal System

Reactions of the ocular glands include atrophy, proliferative and inflammatory lesions. Malfunction results in either excessive or insufficient secretory activity.

2.3.9 Extraocular Muscles

Characteristic lesions include atrophy, necrosis and myositis (inflammation). Malfunction of extraocular muscles is manifested by restricted eye movement, strabismus, diplopia, or complete external ophthalmoplegia. It may be a sequel to neurotoxicity, or it may reflect a direct effect of toxins on the muscles. Nystagmus usually indicates an impairment of the central nervous system.

2.3.10 Ocular Teratogenesis

The normal development of the ocular system in the uterus may be disturbed by a variety of toxins. The most common ocular malformations include microphthalmia, anophthalmia, cyclopia, colobomatous defects, optic nerve aplasia, vitreoretinal dysplasia and defects of the lens (aphakia, microphakia) and persistent embryonal structures: pupillary membrane, and hyaloid vessels.

2.4 SPONTANEOUS LESIONS

2.4.1 Conjunctiva and Cornea

Spontaneous corneal lesions include mineralisation or lipid deposits which may occur in rodents, cats and dogs. Various types of corneal dystrophies in cats and dogs have a suspected or proven hereditary aetiology. They may present as erosions, ulcers, stromal deposits, oedema, or bullous keratopathy. An increase in thickness of Descemet's membrane has been observed in aging rats (Weisse, 1992).

2.4.2 Anterior Uvea

The intraocular pressure depends on the extent of aqueous humour production and outflow. There are two routes of outflow: iridocorneal and uveoscleral. Their relative contribution to total outflow varies among species and this may be responsible for species-related differences in changes of intraocular pressure resulting from eye irritation. Species differences in responsive changes of intraocular pressure may be attributed also to different effects of chemical mediators on the pressure regulation in various species. Pathologically increased intraocular pressure occurs in glaucoma. Spontaneous, inherited glaucoma may occur in man, dogs and rabbits, but in rodents, subhuman primates and cats it is very rare. Other spontaneous lesions affecting the anterior uvea include formation of iris cysts, especially in rabbits, and age-related collagenisation of the ciliary body stroma, reported in rats (Weisse, 1992).

2.4.3 Posterior Uvea

Spontaneous lesions include inherited tapetal degeneration (Bellhorn *et al*, 1975) or inherited absence of a tapetum (Grant, 1986) in dogs.

2.4.4 Lens

Spontaneous cataract change is inherited and, or, age-related. In aging rats, posterior polar cataracts are most frequently observed.

2.4.5 Retina

Spontaneous inherited retinal degeneration occurs in many breeds of dogs, cats, rats and mice. It usually affects the rod photoreceptors initially and then progresses to involve the cones and the remaining retinal neurones. A special form of retinal degeneration has been documented in RCS (Royal College of Surgeons, London) rats; the disease is a primary retinal pigment epitheliopathy manifested by a failure of the retinal pigment epithelium to phagocytose shed photoreceptor outer segments. Phototoxic retinal degeneration may influence the interpretation of experimental studies in rodents, especially in albino strains, which are particularly sensitive to light-induced retinal degeneration (Rubin and Weisse, 1992). Retinal atrophy associated with other changes related to aging may complicate the interpretation of induced effects as well (Weisse, 1992). An enhancement of spontaneous retinal atrophy was observed in rats used for blood collection for laboratory analysis, when large quantities of blood were withdrawn (Krinke *et al*, 1988).

2.4.6 Optic Nerve

In laboratory animals, spontaneous lesions of the optic nerve are uncommon.

2.4.7 Eyelids

Spontaneous drooping of the upper eyelid (ptosis) may occur with advancing age especially in dogs, as well as sagging (turning outward) of the lower eyelid (ectropion).

2.4.8 Lacrimal System

Spontaneous diseases of the lacrimal system include keratoconjunctivitis sicca in dogs and viral sialodacryoadenitis in rats. In the lacrimal and Harderian gland of the aging rat there are prominent spontaneous changes which have been repeatedly confused with experimentally-induced lesions.

They include lymphocytic infiltration, cellular pleomorphism, the occurrence of nuclear pseudoinclusions, the presence of Harderian alveoli in the lacrimal gland, and the formation of porphyrin granulomas in the Harderian gland (Krinke *et al*, 1994).

2.4.9 Extraocular Muscles

In laboratory animals, spontaneous lesions of the extraocular muscles are uncommon.

2.4.10 Ocular Teratogenesis

Spontaneous lesions observed in laboratory animals include anophthalmia, microphthalmia, defects of the lens (aphakia and microphakia), persistent pupillary membrane and persistent hyaloid vessels.

2.5 CHEMICALLY INDUCED OCULAR LESIONS

Examples of pathological lesions, their location within the eye tissue and examples of chemicals and pharmaceuticals known to induce these effects are given below (Table 1). When chemical agents are not specified examples may be found in classical textbooks.

Table 1. Ocular lesions induced by chemicals and pharmaceuticals.

CONJUNCTIVA & CORNEA

Induced Lesions	Chemical Examples	References
Chemical burns	Acids or alkalis	Friedenwald *et al*, 1946 Grant & Kern, 1955
Oedema, erosions	Solvents, surfactants, tear gas, local anaesthetics, topical medicaments	Grant, 1986
Keratoconjunctivitis sicca (→'dry eye' syndrome) ie malfunction of lacrimal glands	Beta-blockers (humans, dogs, not rodents) Clonidine	Fraunfelder & Meyer, 1989 Weisse *et al*, 1978
Neurotropic keratitis →epithelial erosions (impaired sensory innervation of cornea)	Agents inducing trigeminal neuropathy, e.g. pyridoxine	Krinke, pers. comm.
Corneal stromal oedema	Colchicine	Grant, 1986
Corneal deposits	Chlorpromazine Chloroquine Epinephrine	Fraunfelder & Meyer, 1989 Reinecke & Kuwabara, 1963
Corneal lipidosis	Amphiphilic cationic agents	Lüllman-Rauch, 1991a
Corneal mucopolysaccharidosis	Glycosaminoglycans	Lüllman-Rauch, 1991a
Acute irritation on contact	Numerous chemical agents	ECETOC, 1992

ANTERIOR UVEA

Miosis	Cholinergic drugs eg pilocarpine	Millichamp 1992b
Iris cysts/iritis on prolonged exposure to miotics	Agents involved in miotic response: neuropeptides, norepinephrine, products of arachidonic acid metabolism (prostaglandins & leucotrienes)	Havener, 1983
Horner's syndrome ie ptosis, miosis & enophthalmos. (Due to impaired sympathetic innervation of the eye)	Sympatholytic agents	Millichamp 1992b
Hippus (rapid, small oscillations in pupil size)	Barbiturates	Fraunfelder & Meyer, 1989
↓Intraocular pressure	Inhibitors of carbonic anhydrase, miotics (parasympathomimetic drugs), adrenergics, beta-blockers, osmotic agents	Fraunfelder & Meyer, 1989 Grant, 1986
↑Intraocular pressure (glaucoma)	Chemicals disrupting blood-aqueous barrier, parasympatholytic drugs (atropine, scopolamine), mydriatics, corticosteroids	Millichamp 1992

POSTERIOR UVEA

Induced Lesions	Chemical Examples	References
Decolouration associated with oedema and necrosis	Ethylenediamines, dithizone, pyrithione, zinc pyridinethione (Dogs)	Millichamp 1992a
Tapetal colour change without histopathology	Vasodilating agents	Rubin, 1974
Tapetal degeneration with discoloration and pigmentation	Beta-blocker SCH 19927 Aromatase inhibitor CGS 14796C Azalide antibiotic CP-62, 993	Schiavo *et al*, 1984 Schiavo *et al*, 1988 Fortner *et al*, 1993

LENS

Cataracts a) Anterior cortical cataracts	Numerous chemicals. Photosensitisers acting with light exposure of appropriate wavelength eg methoxsalen (man & animals)	Grant, 1986
b) Equatorial cataracts	By agents affecting actively mitotic cells at lens equator eg ionising radiation, nitrogen mustard, antimitotic and antineoplastic drugs	Grant, 1986
c) Diabetic cataract	Due to accumulation of sugars exerting osmotic effect. (Similarly from interference with protein synthesis and metabolism)	Millichamp 1992a
d) Others	Chemicals releasing damaging free radicals, some miotics, biogenic amines, opiates, toxic metals, oestrogenic agents	Lazenby *et al*, 1993
Lens deposits beneath anterior lens capsule	Several metals, chlorpromazine	Millichamp 1992a
Lamellated inclusions/ vacuoles → lens opacities in anterior & posterior cortex.	Lipidosis-inducing agents eg chloroquine, chlorphentermine, iprindole	Drenckhahn & Lüllman-Rauch, 1977

RETINA

Induced Lesions	Chemical Examples	References
Oedema due to breakdown of blood-retinal barrier resulting in protein and water leakage into retinal extracellular compartment. (Can lead to retinal detachment)	Naphthalene (rabbit), triaziquone, dithizone, fluoride (rabbit), iminodiproprionitrile, iodopyracet (a radiopaque contrast medium), streptomycin, quinine, tamoxifen	Pirie, 1968 Sorsby and Harding, 1960
Haemorrhage	Anticoagulant drugs, snake toxins, methyl bromide. (Man > animals)	Grant, 1986
Vasoconstriction	Quinine, ergotamine, oxygen & barbiturates	Brinton *et al*, 1980
Vasodilation	Hypoxia, carbon dioxide, amyl nitrate	Millichamp 1992a

RETINA (cont).

Pigmentary retinopathy ie degeneration of retinal pigment epithelium (RPE) with either ↑ or ↓ of pigmentation (pigmented animals)	Imidazo quinazoline (Beagles ie tapetal)	Schiavo, 1972
Retinitis pigmentosa a) Compound concentration in RPE	Quinolines and phenothiazines	Zinn & Marmor, 1979
b) Other RPE toxins	Sodium iodate Sodium azide, aminophenoxyalkanes, diamino-phenoxyheptane, urethane, methoxyflurane, lead, n-methyl-n-nitrosourea	Noell, 1952 Bellhorn *et al*, 1973
c) Inhibition of phagocytosis of rod outer segments	Isoproterenol, forskolin ie ↑cAMP	Hall *et al*, 1993
Retinal photoreceptor degeneration due to		
a) secondary to damage to RPE	See above	
b) damage to outer retinal layers ie toxicity to photoreceptors	2,5 hexanedione (rat), colchicine, vitamin A, hexachlorophene, amoscanate, iodo-and bromo-acetate 2-aminooxy propionic acid derivatives (rat)	Backstrom *et al*, 1990 Clark *et al*, 1982 Towfighi *et al*, 1975 Lee *et al*, 1979
c) alkylation of photoreceptor nuclear DNA in outer nuclear layer	N-methyl-n-nitrosourea	Ogino *et al*, 1993
Retinal neuronopathy ie damage to retinal ganglionic cells	Doxorubicin, l-cysteine, glutamate (mice) Anthelmintics Monosodium glutamate	Olney, 1969 Low *et al*, 1985 Van Rijn *et al*, 1986
Degeneration with pyknosis in inner and outer nuclear layers	Trimethyltin	Bouldin *et al*, 1984
Amacrine cell degeneration and oedema of inner plexiform layer	Kainic acid	Lessell *et al*, 1980
Retinal lipidosis ie lamellated inclusions in RPE, ganglion cells, Müller's cells and neurones of inner nuclear layer	Cationic amphiphilic drugs eg chlorphentermine, triparanol, l-chloroaminotriptyline, chloroquine	Lüllman-Rauch, 1974 Lüllman-Rauch, 1991**b**

OPTIC NERVE

Induced Lesions	Chemical Examples	References
Papilloedema ie swelling of optic nerve fibres	Methanol	Sharpe *et al*, 1982
	Salicylanilide	Brown *et al*, 1972
Myelinopathy ie myelin damage	Demyelinating compounds eg hexachlorophene, triethyltin	Grant, 1986

OPTIC NERVE (cont).

Axonopathy ie axon damage (most evident in terminal portion of optic tract at surface of lateral geniculate body)	Hexacarbon solvents, clioquinol	Krinke *et al*, 1979

EYELIDS

Ptosis (drooping of upper eyelid)	Agents impairing neurotransmission eg snake toxins, Botulinus toxin, sympatholytic, muscle relaxing or sedating drugs. Also associated with toxic neuropathy	Fraunfelder & Meyer, 1989
Retraction of upper eyelid	Sympathomimetic drugs	Grant, 1986
Blepharitis (inflammation of eyelids)	Various topical agents	Fraunfelder & Meyer, 1989

LACRIMAL SYSTEM

Induced Lesions	Chemical Examples	References
Harderian gland ↑ activity and excess porphyrin production	Dietary deficiency of pantothenic acid Hypervitaminosis A (eventually leading to exhaustion & degeneration)	Krinke *et al*, 1994
Degeneration of Harderian gland due to suppression of thyroxin levels	Herbicide TOK (2, 4-dichlorophenyl-p-nitrophenyl ether)	Gray *et al*, 1982
Harderian gland depletion and acceleration of age-related lesions	Organophosphorus compounds causing prolonged parasympathetic stimulation (Carcinogenicity studies in rodents)	Krinke, pers. comm.
↑ Activity	Associated with corneal/conjunctival irritation	Millichamp 1992a
↓ Activity	Parasympatholytic drugs	Millichamp 1992a
Acceleration of aging changes eg pleomorphism, presence of Harderian alveoli in the lacrimal gland	dl-ethionine, n-2-fluorenylacetamide	Benson, 1964
Degeneration of lacrimal gland → keratoconjunctivitis sicca	Phenazopyridine, sulphadiazine or salicylazosulphapyridine (Dog)	Bryan & Slatter, 1973

EXTRAOCULAR MUSCLES

Myopathy	Anticonvulsants, sedatives, antibiotics, aminoquinolines, organophosphorus compounds, lead and thallium Trichloroethylene (man)	Feldman *et al*, 1970

OCULAR TERATOGENESIS

Various malformations	Selenium, vitamin A, apholate, griseofulvin, chlorambucil, alloxan, benomyl, caffeine, ethanol Nickel carbonyl (rat) Butylated hydroxytoluene (rat)	Hoogenboom *et al*, 1991 Chiou, 1992 Johnson, 1965

SECTION 3. OCULAR TOXICITY OF AGROCHEMICALS AND INDUSTRIAL CHEMICALS

3.1 INTRODUCTION

In this section data are reviewed to provide selected reports of toxic effects induced by xenobiotic chemicals in the visual system of man and laboratory animals. Emphasis is placed on effects caused by agricultural and industrial chemicals. The aim has not been to provide an exhaustive listing of all oculotoxic chemicals. Such comprehensive reviews are already available (Grant, 1986; Millichamp, 1992a). Rather, the aim has been to present examples of chemicals which provide a spectrum of toxic effects against which a strategy for safety assessment for oculotoxic effects can be formulated. Particular attention is paid to the presence or absence in experimental species of effects similar to those observed in man, and to the accuracy of extrapolation from animal investigations to the human situation.

Quantitative extrapolation to a situation of actual human exposure is often difficult because of unrealistically high doses used in animal toxicity studies. However, for the purposes of this report, dosimetry data have been confined largely to studies involving organophosphorus compounds and included in the relevant tables. Dosimetry data on other chemicals can be found in the original references.

3.2 AGROCHEMICALS

3.2.1 Ocular Effects of Organophosphorus Compounds.

Although organophosphorus compounds have been used safely for many years, examples of toxic syndromes have been described.

Acute Syndrome

In man, the appearance of ocular signs of acute toxicity immediately after localised or systemic absorption of organophosphorus (OP) compounds is well documented (Murphy, 1986; Abou-Donia and Lapadula, 1990). Miosis and blurred vision and spasm of ciliary muscle are largely attributable to inhibition of acetylcholinesterase (AChE) at cholinergic (muscarinic) receptor sites within the eye globe, whereas signs such as palpebral tremor observed following acute OP intoxication may be

related to inhibition of AChE at extraglobar receptor sites. For the most part the biological effects of acute exposure to OP compounds in man can be induced in a range of animal species.

Intermediate Syndrome

Subsequent to the acute cholinergic manifestations of OP toxicity in man and approximately 24 to 96 hour post-exposure, onset of an 'intermediate syndrome' which includes ocular effects has recently been recognized (Senanayake and Karalliedde, 1987; Karademir *et al*, 1990). Agents which have been linked with this intermediate syndrome are fenthion, monocrotophos, dicrotophos, dimethoate, methamidiphos, malathion, diazinon and trichlorfon. The associated clinical signs, which are characteristically different from those seen in organophosphorus-induced delayed (poly)neuropathy (OPIDN), are paralysis and weakness of proximal limbs, respiratory, neck and cranial muscles, including those innervated by the oculomotor nerve. Electromyographic studies showed changes suggestive of a post synaptic defect.

Clinical signs of paralysis in hens within 24 hours of dosing some OP compounds (ronnel, methyl parathion, malathion) (Witter and Gaines, 1963), and the occurrence of myopathy in rats exposed to diisopropylfluorophosphate, paraoxon or soman (Wecker *et al*, 1978; Vanneste and Lison, 1993) resembled the features of the "intermediate syndrome" (Senanayake and Karalliedde, 1987). The severity and duration of the myopathy in rats appeared directly related to the degree of inhibition of AChE. Nevertheless, of the compounds which induce this "intermediate syndrome" in man, only malathion has been shown to cause a similar syndrome in animal species.

Long Term Effects

Prolonged exposure to OP's has been associated with the entities known as "Saku Disease" (Ishikawa, 1971, 1973, 1978; Ishikawa and Ohto, 1972; Ohto, 1974; Ishikawa and Miyata, 1980; Dementi, 1994) and "macula changes" (Misra *et al*, 1985). While the validity of certain earlier reports has been questioned (Plestina and Piukovic-Plestina, 1978; Imai, 1986) other reviewers (Dementi, 1994; Boyes *et al*, 1994) have concluded that the overall findings suggest a probable association between human exposure to certain OPs and chronic ocular effects.

The scepticism about the genuine nature of these "diseases" appears warranted, because the incriminated compounds have been used extensively in other parts of the world without causing ocular disease, and common ocular diseases were detected in some of these patients. The epidemiological features of both entities were characterised by their restriction to a fraction of the population in certain geographic areas and a considerable variety of observed signs and symptoms.

The major ocular signs of "Saku Disease" were reduced vision, a narrowing of the peripheral visual field and/or central scotoma, and abnormal refraction or myopic tendency with or without vertical corneal astigmatism. The difficulty in defining "Saku Disease" has led the Japanese Ministry of Public Welfare to list the following diagnostic criteria:

impaired vision (less than 1.0 dioptre after correction),
visual field stenosis (less than 40° nasal and 60° temporal),
progressive myopia,
serious astigmatism (difference between horizontal and vertical radius larger than 2.0 dioptres),
impaired sense of balance (standing time on one leg less than 5 seconds),
effective oxime and atropine therapy,
lowered blood cholinesterase activity,
oedema and atrophy of the optic nerve (pale in colour in the temporal side of papilla),

for which at least five had to be present to be considered compatible with diagnosing the disease (Plestina and Piukovic-Plestina, 1978). The clinical signs associated with "Saku Disease" and their incidence in a cohort of 71 selected patients (Ishikawa, 1973) are summarised below (Table 2).

Table 2 **Principal clinical signs and laboratory findings in 71 Japanese children reported to be affected with "Saku Disease" following chronic organophosphate exposure** (taken from Ishikawa, 1971, 1973; Ishikawa and Miyata, 1980)

Clinical signs	Incidence
Ocular:	
reduced visual acuity refractive abnormalities (myopia)	98%
narrowed visual field	88%
	95%
Central/peripheral neurological[1]:	
dysmetria	71%
peripheral neuropathy (sensory,proprioceptive signs)	
abnormal EEG	
Laboratory Findings:	
OPs in blood or urine > 0.01 ppm	100%[3]
lowered serum cholinesterase[2]	33%

1 Additional autonomic signs reported but incidence not quoted
2 Biopsy of several of these patients indicated total inhibition of cholinesterase activity
3 % of those examined

This table shows that although most of the patients had ocular signs, neurological signs were less frequent and lowered serum cholinesterase was demonstrated in selected cases.

Ocular and other central and peripheral neurological effects were reported, including total inhibition of cholinesterase in biopsies of lateral rectus muscle (Ishikawa and Ohto, 1972) as well as abnormalities of retinal pigmentation. Long term follow-up of 12 of the patients (Ohto, 1974) indicated that most had altered electroretinograms, two being non-recordable, and fundoscopic examination also apparently confirmed retinal pigmentary degeneration, papilloedema and retinal vasoconstriction. Perifoveal hypopigmentation of the retina was the most consistent ocular defect in 19% of the 79 chronically fenthion-exposed pesticide workers in India (Misra *et al*, 1985).

The major OPs used in the period up to 1971 were malathion, O-ethyl-O-P nitrophenyl benzenphosphoro-thioate (EPN), ethyl-and methyl parathion. These were replaced by fenthion, dipterex, fenitrothion, and diazinon, as the latter group was considered to be less toxic to man. All of these compounds have been suspected to be associated with the development of "Saku Disease" (Ishikawa and Miyata, 1980). A list of the principal OP compounds associated with ocular effects in human beings and animals is shown in Table 3. Two carbamate compounds, Hopcide and BPMC, used in association with organophosphorus compounds were considered unlikely to be involved (Ishikawa, 1973). When this list of OP compounds which were allegedly associated with the development of 'Saku Disease' is compared with the list of OP agents capable of producing OPIDN in man, shown in Table 4 (Lotti, 1992), there is only a limited overlap between the two groups of chemicals. This implies that the mechanism of OPIDN is not that involved in ocular effects, attributable to "Saku disease". The findings of visual impairment in man allegedly exposed to OPs have prompted the development of animal models for ocular toxicity induced by OPs. An overview of animal models developed to reproduce particular features of "Saku Disease" is shown in Table 5.

Reduced visual activity associated with myopia and astigmatism in man (Ishikawa, 1971, 1973; Ishikawa and Miyata, 1980) was compared to similar findings in disulphoton- or fenitrothion- treated dogs (Tokoro *et al*, 1973; Suzuki and Ishikawa, 1974; Ishikawa and Miyata, 1980). Degeneration of ciliary muscle reported in these dogs was obviously related to myopia. Also a reduction in cholinesterase activity and the occurrence of degeneration in the extraocular muscle of disulfoton-treated dogs (Mukuno and Imai, 1973) resembled similar findings in man (Ishikawa and Ohto, 1972).

Table 3 Principal organophosphorus compounds associated with ocular effects in man and animals.

Organophosphorus compound	Evidence for ocular effects		
	Man		Animal
	"Saku Disease"	Other	
Fenthion (MPP)	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980	India, macula changes (Misra *et al*, 1985)	Rat (Imai, 1974; 1975, 1977; Miyata *et al*, 1973, 1974, 1979; Imai *et al*, 1983; Uga *et al* 1979; Boyes *et al*, 1994; Mouse (Carricaburu *et al*, 1981)
Fenitrothion (MEP)	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980		Dog (Ishikawa and Miyata, 1980)
Malathion	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980	California case report (Dementi, 1994; Boyes *et al*, 1994)	Mouse (Carricaburu et al, 1981)
Parathion	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980		Rat (Zendzian 1984, 1986, 1987, 1989)
Parathion methyl, Dochlorvos (DDVP), Diazinon, Dioxabenzofos, O-ethyl-O-P nitrophenyl benzenphosphorp-thioate (EPN), Formothion, Trichlorfon (DEP), Vamidothian	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980		
Disulfoton (ethylthiodemeton)	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980		Dog (Araki *et al*, 1973; Hikita *et al*, 1973; Mukuno and Imai, 1973; Mukuno *et al*, 1973; Tokoro *et al*, 1973; Suzuki and Ishikawa 1974; Uga *et al* 1976, 1977a, b)
Mevinphos	Ishikawa, 1971; 1973; Ishikawa and Miyata, 1980		Mouse (Carricaburu et al, 1981)

Table 4 Organophosphorus compounds reported to cause OPIDN in man (from Lotti, 1992).

OP	Number of cases	Circumstances	References
Mipafox	2	Occupational	Bidstrup *et al*, 1953
TOCP	Thousands	Food contamination	Smith *et al*, 1930; Smith and Spalding, 1959; Senanayake and Jeyaratnam 1981 ;Inoue *et al*, 1988
Trichlornat	2	Suicide	Jedrzejowska *et al*, 1980
Parathion	1	Suicide	de Jager *et al*, 1981
Chlorpyrifos	1	Suicide	Lotti *et al*, 1986
Methamidophos	>20	Suicide and occupational	Senanayake and Johnson 1982
Trichlorfon	Several	Mostly suicide	Shiraishi *et al*, 1983 Vasilescu *et al*, 1984 Csik *et al*, 1986
Dichlorvos	3	Suicide	Vasilescu and Florescu, 1980 Wadia *et al*, 1985
EPN	2	Occupational	Xintaras & Burg. 1980
Leptophos	9	Occupational	Xintaras *et al*, 1978.

Table 5 Summary of key results from laboratory animal studies of ocular effects of organophosphorus compounds (from Dementi, 1994).

Compound	Species	Protocol	Key results	Reference
Disulfoton (ethylthiode-meton, ethyl-thiometon)	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Myopia, all dogs > 1yr. Morphological changes in ciliary muscle at all dose levels.	Suzuki and Ishikawa, 1974
	Dog (Beagle)	1mg/kg/day for 2 yr.	Ciliary muscle degeneration. Hypertrophy of basement membrane. Abnormal appearance of mitochondria.	Araki *et al*, 1973.
	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Myopia after 2 years dependent on reduction in erythrocyte ChE activity. Astigmatism > 4m.	Tokoro *et al*, 1973
	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Extraocular muscle and erythrocyte ChE activity reduced, and muscle degeneration. Abnormal appearance of mitochondria and of neuromuscular endplate .	Mukuno and Imai, 1973
	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Retinal degeneration. Optic nerve - reduced number of axons, gliosis.	Uga *et al*, 1976, 1977**a,b**

Compound	Species	Protocol	Key results	Reference
Disulfoton (ethylthiode-meton, ethyl-thiometon) (cont.)	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Demyelination of nerves to an extra-ocular muscle. Reduced number of nerve fibres in optic nerve.	Mukuno *et al*, 1973
	Dog (Beagle)	0.5, 1.0, 1.5 mg/kg/day for 2 yr.	Extraocular muscle and retinal ChE activities reduced at all dose levels.	Hikita *et al*, 1973
Fenthion	Rat (Wistar)	0.005, 0.05, 0.5, 5, 25, 50, 100, 500 mg/kg single sc dose	ERG - 0.005-5 mg/kg supranormal, 25 mg/kg transitional, 50-100 mg/kg subnormal 4 day post dosing. ChE - 0.05 mg/kg NOEL,0.5-100 mg/kg dose related reductions in retina and cerebellum.	Miyata *et al*, 1973; Imai, 1974
	Rat (Wistar)	5, 25, 50 mg/kg single sc dose	ERG - 5 mg/kg supranormal. 25 mg/kg transitory, 50 mg/kg subnormal. Retina and serum ChE activities reduced.	Imai, 1975
	Rat (Long Evans)	50 mg/kg every 4d for 1 yr.	Deaths (9/20). ERG - 3m reduced, 9m a-wave absent, 12m no a- or b-wave. Fundoscopy - vascular constriction. Loss of several retinal cell layers.	Imai, 1977; Miyata *et al*, 1979; Uga *et al*, 1979
	Rat (Wistar, Long Evans)	50 mg/kg sc 2/week for 1 yr.	ERG - reduced at 3m absent at 12m, Retinal degeneration at 12m, 8/12 Wistar and for all Long Evans. Pigmented (Long Evans) more sensitive than albino (Wistar). ChE-erythrocytes and plasma reduced at 3 and 12m.	Imai *et al*, 1983
	Rat (Long Evans)	0.005, 0.5, 50, 100 mg/kg single sc dose	ERG - no effect at any dose. ChE - Retinal, brain, erythrocytes reduced at 50, 100 mg/kg. (cf Imai, 1974).	Boyes *et al*, 1974
	Rat (F344)	0, 5, 40, 100 ppm for 2 yr.	ERG - males unaffected, female dose related b-wave reduction at 18m, 100 ppm females no a- or b-wave at 24m. NOEL 5 ppm. Retinal degeneration - 100 ppm females. Optic nerve axon loss, gliosis in most severe cases. Corneal dystrophy. ChE - >3m, Plasma, erythrocytes, brain <80% depression in females.	Stuart, 1992

Compound	Species	Protocol	Key results	Reference
Fenitrothion	Dog (Beagle)	1, 2, 10 mg/dog, twice per wk for 1 yr, killed 1 yr post-treatment	Myopia - significant in all treated dogs at 9m and 1 yr. Lens - increased thickness after cessation of dosing. Intraocular pressure - increased. Ciliary muscle degeneration. Ciliary muscle ChE activity reduced 1 yr after cessation of dosing.	Ishikawa and Miyata, 1980
Mevinphos and Malathion	Mouse (Swiss White)	Malathion 300 mg ip; Mevinphos 0.015 mg ip.	ERG (both compounds) - reduced a and b waves with up to 40 minute lagtime at peri-lethal doses.	Carricaburu *et al*, 1981
DEF (Tribufos)	Rat (F344)	0,4, 40, 320 ppm for 2yr.	ERG - reduced a- and b-wave in 320 ppm males and females at 24m (not done at 12m). Retinal degeneration at 320 ppm in males and females at 12m and 24m. Cataract, corneal neo- vascularization also present at 24m.	Stuart, 1992
Parathion	Rat	0, 0.5, 5, 50 ppm	Plasma ChE activity reduced at 5, 50 ppm; brain ChE activity reduced at 50 ppm in both sexes. Retinal atrophy - 50 ppm females.	Zendzian, 1984, 1986, 1987, 1989
		0, 2, 8, 32 ppm	ChE NOEL - plasma and erythrocyte activities 2 ppm, brain activity 8 ppm. ERG - decreased at 8 and 32ppm (females). Retina, optic nerve effects at 32 ppm.	

Retinal changes reported in man e.g. retinal pigment degeneration (Ohto, 1974; Misra *et al*, 1985) were also paralleled by similar retinal changes in disulfoton-, fenthion-, tribufos- or parathion-treated rats or dogs (Uga *et al*, 1976, 1977a,b; Imai, 1977; Miyata *et al*, 1979; Uga *et al*, 1979; Zendzian, 1984, 1986, 1987, 1989). Likewise, the electroretinographic (ERG) defects reported in man were also reported in OP-exposed Wistar and Fischer 344 rats or Swiss white mice (Miyata *et al*, 1973; Imai, 1974, 1975, 1977; Miyata *et al*, 1979; Uga *et al*, 1979; Carricaburu *et al*, 1981; Imai *et al*, 1983; Stuart, 1992), but were not reproduced in Long Evans rats following a single dose of fenthion over an extensive range of treatment levels (Boyes *et al*, 1974).

Recently, in two-year rat carcinogenicity bioassays with fenthion and tribufos (DEF) (Stuart,1992) ocular effects have been observed. Besides changes in the ERG and morphology of the retina, dietary exposure in rats was also associated with an increased incidence of corneal dystrophy and cataract with uveitis in one or both sexes at the high dose. Such changes observed in chronic rodent studies may reflect for the most part species-specific senile changes which are not relevant

to the human situation or they may reflect possible chronic dysfunction of ocular structures which are innervated through cholinergic pathways, e.g. changes in secretory activity of the Harderian and lacrimal glands. The adnexa have a secretory function, a failure of which may facilitate development of secondary lesions in the eye. An interference with, or exacerbation of spontaneous, age-related changes in chronic rodent studies are considered not to be a suitable model for chronic human exposure.

There are only limited data available on the level of OP exposure and the extent of absorption in human beings with allegedly induced ocular changes. Serum cholinesterase activity was inhibited by about 26% and erythrocyte acetylcholinesterase activity by about 30% in the study on pesticide workers in India (Misra *et al*, 1985). In the "Saku" study involving 71 patients, serum cholinesterase activity was inhibited by about 22% and erythrocyte acetylcholinesterase activity by about 24% (Ishikawa and Ohto, 1972). Some of these patients showed a complete absence of cholinesterase activity in the lateral rectus muscle; the biological significance of this finding is unclear. Inhibition of blood cholinesterase activity of up to 30%, however, has been considered as a biological threshold limit without risk of toxic effects for man (Gage, 1967; WHO, 1987). Elevated blood levels of selected OPs, e.g. parathion, were observed in some Japanese patients and showed an apparent correlation with the intensity of clinical signs (Ishikawa and Miyata, 1980). 4-nitrophenol, a normal constituent in urine and a known metabolite of certain OPs, was elevated in selected exposed individuals (Ohto, 1977). Although convincing evidence for a causal relationship has not been established, there was at least an apparent coincidence between exposure to certain OPs and the occurrence of ocular changes in these patients.

Although the pathogenesis of "Saku Disease" has not been clarified in detail, there is no reason to presume that mechanisms other than chronic cholinesterase inhibition were involved. In order to test the validity of this presumption, an experimental animal model would be needed, in which chronic stimulation of cholinergic receptors without chemical agents or with chemicals known not to exhibit ocular toxicity would be used. Some authors have suggested that cholinesterase inhibition alone does not explain all ocular effects observed and other mechanisms may be involved. Boyes *et al*, (1994) argue that the major cholinergic neurotransmission in the retina is associated with synaptic contact between amacrine and ganglionic cells and "this activity would not be represented by a-wave or b-wave of ERG". Their view, however, is contradicted by the notion that the ERG, especially the b-wave changes in response to administration of acetylcholine or its agonists (Dementi 1994). Reports on ERG changes induced with very low OP (fenthion) levels, below those that inhibited brain and retinal cholinesterase must be corroborated (Dementi, 1994): they could

reflect a low sensitivity of cholinesterase activity assay. Recent studies on modulation of OP-sensitive muscarinic receptors indicate that selected OPs may have different, possibly direct, effects on the receptors (Chaudhuri *et al*, 1993). Furthermore, it has been suggested that metabolites of some OPs may exert toxicity which differs from the parent molecule (Abou-Donia *et al*, 1979); however, this suggestion can only be substantiated by conducting further studies. Obviously, more fundamental research will be needed before any conclusive statements on other possible mechanisms for ocular effects of particular OPs can be made and practical recommendations for their testing formulated.

3.2.2 Ocular Effects of Other Agrochemicals

There are no reports of ocular effects associated with diquat exposure in man but dietary administration of this herbicide (1,1'-ethylene-2.2'-dipyridylium dibromide) to dogs and rats has led to the development of cataracts after 4-8 months (Pirie & Rees, 1970, Pirie *et al*, 1970). Initially posterior subcapsular cataracts developed which progressed to involve the nucleus and later the entire lens in the rat. The mechanism by which these cataracts develop is unclear. Whilst light is essential to the development of cataracts with several cataractogenic agents, rats fed diquat and kept in complete darkness still developed cataracts. A free radical mechanism has been postulated. Scavengers of free radicals include glutathione and ascorbic acid and it may be significant that ascorbic acid is depleted in the lens of rats fed diquat (Pirie *et al*, 1970). Since prolonged administration of diquat is required to develop cataract, it is most unlikely to occur in man, given the use pattern of diquat and the low levels of occupational exposure.

Amitrole and other triazoles can also induce cataract in laboratory species such as the rabbit or Beagle dog (Bhuyan *et al*, 1973; P. Berry, pers. comm.). In the case of rabbits treated with amitrole, cataract induction after 2-4 weeks exposure was associated with 83 per cent inhibition of lens catalase and 2-3 fold increases in aqueous humour peroxide concentrations. A resultant free radical excess may be the prime factor in cataract induction by this compound.

Dichloran (2,6-dichloro-4-nitroaniline) has been used to inhibit growth of mould on foods. Chronic feeding of dichloran leads to corneal opacity in dogs exposed to sunlight but not in those kept under dark conditions (Earl *et al*, 1971). Ophthalmoscopic examination showed corneal changes in the anterior corneal stroma just below the epithelium. The lesion was not reversible following cessation of treatment and a prolonged recovery period. In contrast, miniature swine fed identical concentrations of dichloran for long periods showed no similar ocular lesion.

Benomyl (methyl 1-butylcarbamoyl-1-benzimidazole carbamate), a systemic fungicide, which has been shown to produce ocular malformations in foetal rats when administered orally to the dams between days 7-21 of gestation. These malformations include microphthalmia (Kavlock *et al*, 1982), anophthalmia (Zeman *et al*, 1986), cataracts and retinal dysplasia (Delatour and Richard, 1976: Hoogenboom *et al*, 1991). Histologically, the retinal dysplasia was characterised by rosettes of retinal cells and retinal folding. Benomyl is known to bind specifically to tubulin and prevent microtubule formation in the brain. The anti-tubulin action of benomyl may offer an explanation for the brain and ocular malformations by disruption of neuronal proliferation and integration.

3.3 SOLVENTS

3.3.1 Ocular Effects of Methanol

Because of the frequent occurrence of methanol intoxication in man (Benton and Calhoun, 1952; Sejersted *et al*, 1983) and the potential for use of this chemical as a major automotive fuel in the next century (Kavet and Nauss, 1990), the well known oculotoxic effects of methanol have been investigated in some detail. Initial visual symptoms of intoxication in man may be blindness or serious visual impairment including diminished pupillary reflex within 18-48 hours of ingestion. Ophthalmoscopy may reveal hyperaemia of the optic disc followed by striated oedema of the disc margins and adjacent retina persistent for 10-60 days. Atrophy of the optic disc may be discernible from 30-60 days after exposure. Electroretinographic analyses (Ruedemann, 1961, 1962; Eells *et al*, 1991) have shown reductions principally in the scotopic b-wave amplitude which precede the development of optic disc oedema. Morphological findings including retinal ganglion cell degeneration reported in man are of uncertain significance because of the possibility of post-mortem artefact (Benton and Calhoun, 1952; Hayreh *et al*, 1977, Sharpe *et al*, 1982). However, recent light and electron microscopic examination of immersion-fixed eyes taken immediately following death from a fatal case of methanol poisoning indicated mitochondrial disruption, swelling of retinal pigmented epithelium and preferential destruction of rod cells (Eells *et al*, 1991).

Use of animal models has demonstrated that the toxic metabolite most likely to be responsible for the oculotoxic effects of methanol is formate (Martin-Amat *et al*, 1978; Hayreh *et al*, 1980; Blomstrand and Ingemannson, 1984). Whereas primates accumulate plasma levels of formate above non-toxic threshold concentrations, other species rapidly metabolise it via a tetrahydrofolate-dependent pathway and thus cannot normally be used to model the toxicity. Following oral administration of methanol to monkeys (Potts *et al*, 1955, Hayreh *et al*, 1977, 1980) the clinical signs reported, including reduced pupillary reflex, are similar to those seen in man. Fundic oedema

occurs which may involve the optic disc alone, or variably affect the retina to include the entire fundus. Electroretinographic changes include a reduced b-wave amplitude and variable effects on the a-wave (Potts *et al*, 1955, Ingemansson, 1983). In immersion-fixed tissue, morphological changes reported included vacuolar degeneration of the external nuclear layer of the retina (Potts *et al*, 1955), but in perfusion-fixed eyes, changes were confined to myelinated axons and astrocytic processes in the enlarged (oedematous) prelaminar and retrolaminar optic nerve (Hayreh *et al*, 1980).

An additional animal model of methanol intoxication has recently been developed in the Long Evans rat (Eells, 1990; Murray *et al*, 1991). In this model, continuous exposure to subanaesthetic concentrations of nitrous oxide blocks methionine synthase leading to the depletion of tetrahydrofolate and accumulation of the toxic metabolite formate after methanol exposure. Visual dysfunction evidenced by reductions in the flash evoked cortical potential as well as the electroretinographic a- and b-waves parallels blood formate accumulation. Electron microscopic examination of perfusion-fixed tissue showed mitochondrial disruption which accompanied extracelluar vacuolation of the retinal photoreceptor layer and infolding of the pigment epithelium as well as axonal swelling of the optic nerve.

3.3.2 Ocular Effects of Carbon Disulphide

This is a highly volatile solvent used widely in industry, particularly in the manufacture of viscose rayon. The clinical manifestations following exposure to carbon disulphide are diverse and include behavioural disorders and peripheral neuropathy (NIOSH, 1984). Impaired vision is a common feature in human beings occupationally exposed to carbon disulphide, although many of the reported findings appear to lack specificity. A disturbance of the pupillary reaction to light has been described (Savic, 1967); however, this phenomenon has only been seen at unusually high concentrations of carbon disulphide exposure. An epidemiological cross-sectional survey has described an increased incidence of colour vision deficits in viscose rayon workers (Raitta *et al*, 1981). The deficits did not match those typical of congenital colour deficiencies. However, in two other epidemiological studies involving exposure to carbon disulphide, no differences in colour vision and acuity were detected when compared to age-matched control groups (De Laey *et al*, 1981; Putz-Anderson *et al*, 1983).

Retinal vascular changes have been reported to be an especially sensitive indicator of early toxicity (Raitta & Tolonen, 1980). Visual loss occurred in adult pigtailed macaque monkeys exposed by inhalation to carbon disulphide for 7 weeks and was most pronounced on visual acuity. In contrast,

the monkeys showed only slight and transient impairment of flicker resolution. No retinal vascular abnormalities were detected at the end of the exposure period (Merigan *et al*, 1985; Merigan *et al*, 1988). Visual loss was found to be only partially reversible with marked degeneration of central retinal ganglion cells with little or no effect on other neurones in the retina (Eskin *et al*, 1988).

3.3.3 Ocular Effects of Other Solvents

Systemic administration of 1,2-dichloroethane causes corneal clouding specifically in the dog; however, its lethality is approximately equivalent in cats, dogs, rabbits and rats (Kuwabara *et al*, 1968). When administered into the anterior chamber of the eye, it is equally toxic to the cornea of these species, causing endothelial necrosis. Thus the apparent vulnerability reflects the amount of substance contacting the endothelium rather than an unusual susceptibility of dog eye.

Dimethylsulphoxide (DMSO) is a highly polar organic solvent with widespread use. Lens abnormalities have been reported in several animal species, including dogs, rabbits and swine following either oral or dermal exposure (Rubin and Barnett, 1967; Wood and Wirth, 1969). The ocular abnormality found in experimental animals is a change in the transparency and refractive power of the lens. True lens opacities and cataracts have been reported in dogs and rabbits only after subchronic administration at very high dose levels (> 1 g/kg/day). Lower concentrations of DMSO when administered to man over long periods have caused no adverse effects on the eyes (Gordon and Kleberger, 1968; Bernstein, 1977).

There is growing evidence that links organic solvent exposure with acquired colour vision loss (Mergler *et al*, 1987; Mergler, 1990). This may result from changes in ocular structures such as lens opacification or from altered functioning of the neuro-optic pathways and is considered to be a sensitive indicator of the severity of optic neuropathy.

Chronic occupational exposure to n-hexane is known to cause peripheral neurotoxicity. In addition, there is evidence of toxicity to the ocular system manifested as blurred vision, with optic atrophy and retrobulbar necrosis. In experimental animals, visual loss, abnormal pupillary reflexes and nystagmus have been reported (Schaumburg & Spencer, 1976; Spencer & Schaumburg, 1977, 1985). Administration to rats of the related neuropathic hexacarbon compound, 2,5 hexanedione, for up to 5 weeks caused retinal changes characterised by reductions in the outer nuclear layer, and outer and inner segments of rods and cones (Backstrom *et al*, 1990).

3.4 TOXIC METALS

Visual disorders have been attributed to exposure to several toxic metals (Grant 1986). Vision deficits in both human and non-human primates have been found after exposure to inorganic lead or organic mercury and attributed to a reduction in the rod-mediated, scotopic visual function as opposed to the cone mediated, photopic, visual function. The prevalence of visual deficits arising from methylmercury exposure is believed to be due to its preference for the cerebrum, particularly for the visual cortex. Visual evoked cortical potentials (VECP) have been measured in dogs receiving low daily doses of methylmercury orally, for up to 55 days. Subtle changes in the VECP were seen after 7 days of exposure, corresponding to the preclinical 'silent' stage (Mattson *et al*, 1981).

Low concentrations (5-50 μM) of several toxic metals including cadmium, lead and mercury have been shown to reduce the amplitude of the rod receptor potential in the perfused bullfrog retina; in contrast, responses from the cones were not affected (Fox & Sillman, 1979). The effects of cadmium and lead in depressing rod activity are readily and fully reversible (Sillman *et al*, 1982). Mercuric acetate when administered repeatedly to cats every other day for up to 4 weeks caused changes in the electroretinogram pattern. The amplitude of the scotopic b-wave was increased and sustained, indicating a permanent increase in the excitability level of the cat retina (Gitter *et al*, 1988).

Retinal changes have been found in rats following acute administration of trimethyltin (Bouldin *et al*, 1984). Although the degree of pathological changes in retinal neurons was less than in the hippocampus, necrosis was confined selectively to photoreceptor cells and rare neurones in the inner nuclear layer; the retinal pigment epithelium was unaffected.

3.5 OTHER CHEMICALS

Several compounds known to chelate zinc ions have been shown to cause species-specific retinal damage. The species specificity is dependent on the presence of the tapetum lucidum, which is absent from some species including man and non-human primates. Dithizone (or diphenylthio-carbazone) when administered acutely to rabbits produced chorio-retinal lesions visible microscopically after 8 hours and ophthalmoscopically after 12 hours. Even earlier changes were detected by electroretinography since the b-wave diminished rapidly and was abolished in 3-4 hours (Babel & Ziv, 1957). Zinc pyridinethione has been shown to cause blindness and retinal

detachment in dogs (Snyder *et al*, 1965). Both dithizone and hydroxypyridinethione have also shown evidence of retinal detachment in dogs (Delahunt *et al*, 1962).

In a species comparison study in which zinc pyridinethione was administered orally to cats, monkeys and two groups of Beagle dog (one with a tapetum and the other atapetal), a clear association of a retinal lesion with those species possessing a tapetum was demonstrated (Cloyd *et al*, 1978). Tapetal degeneration and atrophy were seen in the cat and the standard Beagle but no gross or histopathological ocular lesions occurred in the monkey or atapetal dog. Since the tapetum lucidum of carnivores is zinc-rich, this is considered to offer a rational explanation for the tapetum as a primary target site for zinc chelators.

Ethylene oxide is widely used as a fumigant and sterilising agent. Exposure to ethylene oxide has been associated with formation of cataract in man (Jay *et al*, 1982). In an epidemiological study of 55 subjects occupationally exposed to ethylene oxide in six sterilisation units, 19 of these subjects were shown to have lens opacities (Deschamps *et al*, 1990). These findings are of particular concern to hospital workers who use ethylene oxide as a sterilising agent. Although ethylene oxide has been shown to be acutely irritant to the eye of man and animals, in the vapour phase,corneal opacities have only been reported in a single animal species, the guinea pig, at high vapour concentrations (Hine *et al*, 1981). There has been no evidence of major ocular (systemic) effects in animals other than those resulting from direct instillation into the eye (Glaser, 1978).

Repeated anaesthesia of newborn rats with urethane (ethylcarbamate) caused degeneration of photoreceptor and pigmented epithelial cell retinal layers (Bellhorn *et al*, 1973). Ophthalmoscopy and fluorescein angiography revealed early alterations in fundus pigmentation. Histological examination of the retina showed early loss of visual receptor cells. Some aspects of this retinopathy are similar to those described for hereditary retinal dystrophy in pigmented rats.

3.6 EVALUATION

Analysis of the available data leads to the conclusion that a causal relationship between exposure to high levels of some chemicals and impairment of the visual system in man is probable. The evidence is, however, circumstantial and is usually based on retrospective analysis. There are open questions with respect to the pattern of geographical distribution, possible multifactorial etiology or increased susceptibility in certain regions and populations. Additionally it is unclear why

certain agents should be oculotoxic while other agents of the same or related chemical class should not. At present these questions can only be answered by speculation.

The majority of the ocular effects arising from exposure to chemicals in this section have been confined to animal studies. Similar findings are unlikely to occur in man when levels of human exposure are reasonably low. With some of these compounds, such as carbon disulfide or hexacarbon solvents, tragic accidents occurred in the past, before the toxic hazard to occupationally exposed workers was recognized. Ocular effects of these compounds are obviously closely related to their propensity to induce neuropathy, which has been observed during chronic exposure at levels of 20 to 40 ppm for carbon disulphide and more than 100 ppm for hexcarbon solvents. By regulating the "Threshold Limit Value" (e.g. in the U.S.A. to 10 ppm for carbon disulfide and 1 ppm for hexacarbon solvents) this occupational hazard could be appropriately contained (Seppäläinen and Haltia, 1980; Allen, 1980). Such measures, however, can be taken only when a causal relationship between the exposure to a chemical and the toxic effect has been clearly established.

The question of chronic oculotoxic potential of OPs and the need for further regulatory studies remain highly controversial. A number of OPs have already been tested in oral toxicity studies in animal species at levels up to 10,000 fold greater than the reference dose ("no observed effect level" divided by a safety factor), without revealing evidence of oculotoxicity (Katz, 1994). Well conducted animal studies with selected OPs have shown an absence of oculotoxicity (Atkinson *et al*, 1994), and the difficulty in confirming earlier positive findings (Boyes *et al*, 1994). Some studies on exposed human beings in Japan (Mitsumi *et al*, 1975) and elsewhere (Plestina and Piukovic-Plestina, 1978) concluded that no significant ocular effects were to be anticipated from OP exposure. The scepticism about the causative factors of "Saku Disease" is based primarily on the extensive use of OPs worldwide over many years, but with the occurrence of this ocular syndrome confined to a small area of the world only. Moreover, the diagnosis of common ocular disease in some of the patients previously considered as cases of "Saku Disease" (Imai, 1986), casts further doubt on the alleged association with chronic exposure to some OPs.

For obvious ethical reasons man cannot be used for prospective toxicological evaluation and reliance has to be placed on the use of animal experimentation. Extrapolation to man of the ocular effects seen in animals must be conducted with care. There is a difference in the value of the findings from specialised animal models designed to replicate observed human pathology and those of an experimental nature obtained from standard animal safety evaluation studies. The observations from animal models, especially those obtained using OPs, show quite a good

correlation with the effects seen in man. Contrary to this, as evidenced by Fletcher (1978) and Heywood (1981, 1983), the correlation between animal toxicity study data and the occurrence of human eye lesions appears poor as many effects observed in animals may not be relevant for man. False positive animal responses seem to be more frequent than false negative (Table 6) possibly because in animal experiments extreme exposure conditions may be achieved, surpassing by far the exposure scenario experienced by man. False negative animal responses seem to be rare and as a rule they can be attributed to metabolic or morphologic differences. In addition disturbances of several functional or dynamic parameters of vision (e.g. visual acuity, visual field, binocular vision, colour vision) can only be tested in man as their assessment requires a subjective contribution by the test person (so called "subjective" methods).

Table 6 Examples of chemicals and pharmaceuticals manifesting species differences in visual system toxicity in laboratory animals and man.

Chemical	Species differences in visual system response	References
Clioquinol	Optic neuropathy in man and especially dog. Monkey (Baboon) less susceptible Rat not affected.	Krinke *et al*, 1979; Thomas *et al*, 1984; Krinke, pers. comm.
Methanol	Retinal and optic neuropathy in man and non human primates. Other species resistant unless pre-treated (with nitrous oxide).	Eells, 1990; Murray *et al*, 1991
Ethambutol	Tapetum lucidum degeneration in dog not relevant to man. Optic nerve/tract lesions in man, monkey, dog, rat and rabbit.	Lessell, 1976; Grant, 1986
Amoscanate	Retinal degeneration in rat. No effect in dog or monkey. No reported clinical effects in man.	Clark *et al*, 1982; Krinke *et al*, 1983 Schaeppi *et al*, 1987
Lovastatin	Optic neuropathy, cataract in dog. No effect in rat or monkey. No clinical effects in man.	MacDonald *et al*, 1988; Berry *et al*, 1988
Cortico-steroids	Posterior subcapsular cataracts in man but not in animals after systemic application.	Koch, 1977; Kuck, 1977 Potts and Gonasun, 1980
Dimethylsulphoxide (DMSO)	Lens changes in various experimental animals but not in man.	Rubin and Mattis, 1966 Rubin and Barnett, 1967 Gordon and Kleberger, 1968 Bernstein, 1977
Piperidylchloro-phenothiazine (Sandoz NP 207)	Phenothiazine retinopathy in man and in cat but not in albino or pigmented rat, dog, rabbit and guinea pig.	Kinross-Wright, 1956 Meier-Ruge and Cerletti, 1966
2,4-dinitrophenol	Lens opacities in man and chicken, but not in rabbit or dog.	Hockwin *et al*, 1992a

SECTION 4. TEST METHODS FOR OCULAR TOXICITY IN LABORATORY ANIMALS

4.1 INTRODUCTION

The aim of this section is to review the various test methods and their applicability to animals during toxicity studies. Likewise, the post mortem techniques for the preparation of animal eyes and the application of light and electron microscopy are described followed by physiological and biochemical assays. The methods mentioned are separated into routinely used standard procedures as well as extended and specialised methods.

The various techniques are summarised in Table 7. The extended methods may be applied in addition to standard methods on animals from routine toxicity studies, when induced ocular lesions have to be defined and documented in more detail, or are anticipated. The specialised methods require additional animals (satellite groups), special devices, sedation or anaesthesia, experienced professional skills, and are indicated only in special instances when induced ocular findings have to be further characterised or the pathogenesis of the induced lesions has to be studied.

Despite the difficulties in the extrapolation of ocular findings from (albino) animal experiments to man, the ophthalmologist has at his disposal a large number of objective diagnostic procedures for identifying oculotoxicity *in vivo* (Rubin, 1992). Moreover, as a useful and necessary supplement to ophthalmology, histological examination of the eyes represents a second diagnostic route to assess ocular damage. Both the clinical and post mortem methods of eye examination are complementary and should make it possible to recognize unexpected potential oculotoxicity and to prevent eye damage in man (Heywood and Gopinath, 1990; Weisse, 1992).

The biochemical methods are suitable for studying specific effects of selected chemicals and will be used in context when extended or specialised rather than routine approaches are required. The specialised electrophysiological methods yield quantitative data which may be helpful for estimating dose-effect relationships in exceptional situations when data obtained by standard and extended methods must be corroborated or more precisely defined.

4.2. CLINICAL METHODS

Various techniques exist for clinical examination of the eye. The standardised methods are quite easy to perform, whilst the others require special apparatus and specific skills on the part of the observer (Table 7).

Table 7 Clinical and post mortem methods of eye examination in toxicity studies.

Clinical Methods:	STANDARD	EXTENDED	SPECIALISED
Anterior eye segment			
dog monkey rabbit (pig)	- examination with focal illumination - direct/indirect ophthalmoscopy	- slit lamp biomicroscopy - slit lamp photography - external ophthalmic stains - Schirmer tear test - esthesiometry - pupillometry - tonometry	- pachymetry - examination of refraction
rat mouse	- slit lamp biomicroscopy - direct/indirect ophthalmoscopy	- slit lamp photography	- Scheimpflug photography/ biometry
Ocular fundus			
dog monkey rabbit (pig)	- direct/indirect ophthalmoscopy	- fundus photography - fluorescein angiography	- Electroretinography (ERG)
rat mouse	- indirect ophthalmoscopy	- fundus photography - fluorescein angiography	- Electroretinography (ERG) - visual evoked cortical potential (VECP)
Microscopical methods:			
	- light microscopy	- transmission electron microscopy (TEM) - scanning electron microscopy (SEM)	- perfusion fixation - transmission electron microscopy (TEM) - scanning electron microscopy (SEM)

4.2.1 Standard Methods of Clinical Eye Examination

Routine ophthalmic examination techniques in toxicity studies should be easy to perform and applicable to non-anaesthetised animals (Heywood, 1982) because a larger number of animals

must be repeatedly inspected. With the exception of monkeys where the ocular examination requires sedation (0.25 ml/kg ketanest [50 mg/ml] i.m.), the examination in dogs, minipigs, rabbits, rats and mice is generally performed by manual restraint in a light intensity controlled room. These standard procedures are suitable to evaluate clinically the majority of lesions caused by chemical exposure to the eye.

Examination of the Anterior Segment and Adnexa

(The anterior segment of the eye includes the cornea, anterior chamber, iris and lens. The adnexa refers to the conjunctiva, eyelids and lacrimal apparatus.)

Examination with Focal Illumination

In dogs and monkeys the anterior segment including the lens and adnexa can be examined properly with or without a magnifying loupe (2-4 x) and a direct or oblique illumination with a focal beam of light from a hand-held source. Inflammatory lesions, discharge, abnormal content in the anterior chamber or opacities in refractive media can be recognised easily or the pupillary light and palpebral blink reflex can be tested.

Direct or Indirect Ophthalmoscopy

Although these methods in toxicity studies with non-rodents and rodents are used routinely to examine the ocular fundus (see below), both techniques are suitable also for screening of the cornea, anterior chamber, pupillary area and lens. Dilation of the pupils (1% tropicamide) is essential. With the direct ophthalmoscope, held at a distance of 30 cm from the non-rodent eye, or at 5 cm from the rat eye the refractive media can be assessed generally in the light of the fundus reflex. A more detailed examination of the anterior structures can be made with the direct ophthalmoscope held 2 - 3 cm in front of the (non-rodent) eye and by using the rotating plus lenses (the crystalline lens is seen between +8 and +12 dioptres, the iris, the anterior chamber, and cornea between +12 and +20 dioptres). The more preferable indirect ophthalmoscope enables discernment of ocular densities in the refractive media or the anterior chamber. With these two procedures the position of ocular opacities can be determined by their relationship to the eye movement (parallax). When comparing the diagnostic value of direct ophthalmoscopy, indirect ophthalmoscopy and slit lamp biomicroscopy, Bellhorn (1981) established that direct and indirect ophthalmoscopy are less sensitive methods. However, in toxicity studies the routine use of indirect

ophthalmoscopy, in particular, for the assessment of the anterior eye segment is recommended for all species (Heywood, 1985; Rubin, 1992).

Slit Lamp Biomicroscopy

For a closer examination of the anterior eye segment and adnexa, slit lamp biomicroscopy is indicated. The use of a binocular microscope equipped with magnifications from 5 to 40 x and a special flexible illumination system, allowing different illumination techniques, enables a well-illuminated and highly magnified view of the different parts of the anterior eye segment to be made. In particular the use of the optical (slit) section allows a precise localisation and description of abnormalities.

In toxicity studies with rodents, in particular, slit lamp biomicroscopy represents the method of choice for the routine examination of the anterior segment (Taradache and Greaves, 1984; Weisse *et al*, 1987; Hockwin *et al*, 1992a). The pupils must be dilated 10-15 minutes before the examination using 1% tropicamide for the albino and 1% atropine for the pigmented rat. A stationary slit lamp is recommended. Rodents are restrained manually in front of the microscope and the eyelids are everted so that the globes protrude. The adnexa, cornea, anterior chamber, pupillary area and lens can be visualised from the surface inwards with a magnification of 5 to 20 x. Initially short diffuse illumination with a wide beam is used and subsequently focal illumination with a narrow slit is employed.

The examination of the anterior eye segment of rodent eyes in toxicity studies with slit lamp biomicroscopy has the following advantages:

- it magnifies very discrete lesions by a factor of up to 40 x,
- it is superior to light microscopical assessment of the refractive media and especially the lens because the entire cornea or lens can be examined by an optical slit and lens artefacts caused by histological processing are not present.

Based on these advantages one can dispense with histopathological examination of rodent lenses in toxicity studies.

Examination of the Ocular Fundus

Direct Ophthalmoscopy

For adequate visualisation and assessment of the ocular fundus both a darkened room and dilated pupils (1% tropicamide) are necessary. Direct ophthalmoscopy uses the refractive components of the animal eye as a magnifying system. The fundus image is magnified approximately 15-times, is real and erect. Fundus structures smaller than 70 µm (e.g. retinal capillaries) cannot be resolved. Any refractive changes in the animal's and examiner's eye can be corrected by intervening convex or concave lenses. In addition, these rotating corrective lenses permit measurements of elevated or depressed fundus lesions. The closer the examiner is to the animal's eye (2-3 cm), the larger the fundus view will be (about 9-10 °).

Direct ophthalmoscopy as a method has some limitations. Due to the small field of view and relatively great magnification, distortion occurs if the refractive media are translucent. Examination of the temporal fundus parts in most laboratory animals is difficult and the short working distance can be hazardous or inconvenient (e.g. induced salivation in the examined animal). Direct ophthalmoscopy can be used routinely in experimental toxicology (non-rodents), but is a complementary method to indirect ophthalmoscopy, e.g. for proper assessment of the macular and foveal regions and the optic disc in non-human primates (Bellhorn, 1981).

Indirect ophthalmoscopy

As with direct ophthalmoscopy, mydriasis and semi-dark conditions are required. The principle of this method is as follows: the light beam emanating from the indirect ophthalmoscope illuminates the animal's ocular fundus, in part the light rays are reflected back to the examiner's field of view. Interposing a hand-held condensing lens (non-rodents: +13 to +23 dioptres, rodents: +25 to +45 dioptres) about 7-10 cm anterior to the animal's eye results in a virtual, inverted and 2-5 x magnification of the fundus. Objects in the fundus smaller than 200 µm cannot be resolved.

The advantages of this method over direct ophthalmoscopy are that the low magnification and large field of view provide a wider view of the fundus area (about 40° with a +20 dioptres lens). The bright light of the binocular ophthalmoscope penetrates cloudy refractive media better. Despite the inverted and reversed fundus image and the smaller degree of magnification, indirect

ophthalmoscopy is the most common method used for the examination of the ocular fundus in laboratory species.

4.2.2 Extended Methods of Clinical Eye Examination

Extended examination may be required if the initial routine examinations reveal or imply the presence of treament-related lesions. They may be applied on animals from routine toxicity studies, and should be selected to elucidate appropriately the suspected lesions.

Examination of the Anterior Eye Segment and Adnexa

Slit lamp biomicroscopy (non-rodents)

In dogs and monkeys (or rabbits) the biomicroscopic assessment of the anterior eye segment is a second step following a general eye examination with lower magnifications. In contrast, for rodents slit lamp biomicroscopy is the commonly used standard method.

The use of a table-mounted biomicroscope is better than a hand-held instrument. To keep the animal's head in the correct position, restraint is necessary. Sedation or anaesthesia is not recommended. In performing the biomicroscopic examination pupils are dilated and the anterior segment illuminated with a diffuse wide beam to scan the external adnexa and then the ocular media. Subsequently the observed lesions will be examined with focal illumination (narrow slit) or other observation techniques. More detailed methods for the biomicroscopic examination in laboratory animals are given by McDonald *et al* (1973), Hockwin *et al* (1992a) and Rubin (1992).

Slit lamp photography

Slit lamp microscopic photography of the anterior eye segment depends on the image-recording devices available. In contrast to portable slit lamps, table-mounted instruments are suitable for photography. There are several factors which influence the results (e.g. flash intensity, width and length of slit aperture, diaphragm) and therefore the conditions of exposure have to be explored individually. It is advisable to test typical exposure situations with test film(s) before the use of photographic recording in toxicological studies. Lesions of the conjunctiva, cornea or lens can be photographed with diffuse illumination at a magnification of 0.7 to 4.5 x.

The aim of slit lamp photography in experimental toxicology is two fold. It enables qualitative documentation of relevant lesions and their development during the course of the study which is of use for diagnostic interpretation. Additionally it estimates quantitatively the size of the lesion.

With the exception of anticipated lesions (for instance when oculotoxic lesions have occurred in preceding studies using the same chemical compound, or where chemicals with similar structures or similar pharmaceutical activity are known to induce oculotoxicity) there are no convincing arguments for routine photographic documentation of the anterior eye segments at the beginning or end of toxicity studies.

External ophthalmic stains

External ophthalmic stains in experimental toxicology can be essential diagnostic aids in the evaluation of the conjunctival, corneal or preocular tear film integrity. Most frequently used dyes are fluorescein and rose bengal. Apart from the use of fluorescein in eye irritation testing (Baldwin *et al*, 1973, McDonald *et al*, 1983) fluorescein staining can be indicated in systemic studies. In non-rodents particularly, fluorescein staining together with slit lamp biomicroscopy assists in the definition of subtle corneal epithelial defects and in the assessment of preocular tear film stability (break-up time, Moore, 1990). Fluorescein staining can be accomplished either by sodium salt solutions (0.25% - 2%) or preferably, by the use of impregnated paper strips. Damage to the corneal epithelium causes retention of the yellowish-green dye. Interpretation is facilitated by rinsing the eye with an irrigating solution in order to remove non absorbed or excess fluorescein.

Vital staining with the pinkish-red rose bengal (0.5% solution) can be used in non-rodents to detect degenerating or dead cells and mucus, which all stain brilliant red. According to Gelatt (1972), for the correct assessment of rose bengal retention, excess stain (caused by 1 drop of the 0.5% dye solution) should be rinsed after 1 min with 0.9% saline. For reliable assessment of this staining slit lamp biomicroscopy is essential.

The use of both staining techniques can assist in the diagnosis of compound-related keratoconjunctivitis sicca in non-rodents, but not in rodents.

Schirmer tear test

The preocular tear film covering the exposed part of the cornea and bulbar conjunctiva consists of three layers: (1) an innermost glycoprotein layer secreted by the conjunctival goblet cells which acts as a wetting agent for the hydrophobic corneal epithelium, (2) an intermediate aqueous (tear) layer produced by the lacrimal glands which provides corneal nutrition and (3) a superficial lipid layer generated by the tarsal Meibomian glands which prevents evaporation of tear fluid. Disturbances in quantity or components of the tear film result in irritation of the anterior eye structures ("dry eye syndrome").

The diagnostic assessment of glycoprotein and lipid abnormalities in tears of laboratory non-rodents is described in more detail by Moore (1990). With the Schirmer tear test I the tear flow (aqueous layer) resulting from the basal and reflex secretion can be measured by the extent of wetting of filter paper strips (in mm/min or mm/5min) inserted into the lower conjunctival fornix of dogs (Rubin *et al*, 1965; Harker, 1970; Weisse *et al*, 1974). In toxicological studies this simple method can be applied repeatedly to conscious non-rodents, allowing an estimate of the change in tear production. A modified version of this test is also applicable to rats (Weisse *et al*, 1978). Moreover, Thörig *et al* (1983) described a method for measuring the tear production in lightly sedated rats using cotton-threads. For a more detailed investigation of tear production, the Schirmer tear test II can be used, which solely measures the basal tear secretion under local anaesthesia of the cornea and conjunctiva (Rubin, 1992).

Esthesiometry

Increased intraocular pressure, oedema of the corneal epithelium or exposure to chemicals can influence corneal sensitivity in laboratory animals. Corneal sensitivity can be determined easily by the corneal reflex, i.e. gently touching the corneal surface elicits closure of eyelids.

The Cochet-Bonnet esthesiometer, after a little practice, provides quantitative estimation (g/mm by using a conversion table) of the corneal sensitivity threshold in non-rodents. With the aid of a nylon monofilament (preferred type: 0.12 mm in diameter) which can vary in length (up to 60 mm), the pressure applied to the corneal epithelium ranges from 11 to 200 mg/0.0113 mm . Experience shows that in Beagle dogs 30 - 40 mm (1.8 - 3.2 g/mm) and in rabbits 20 - 30mm (3.2 - 6.6 g/mm) are the lengths of monofilament which represent normal threshold values for the induction of the cornea reflex. It is advisable to touch the central cornea repeatedly (e.g. 5 times) with the same

filament length to be sure that stimulation is really sufficient. This method is not suitable for rodents (Krausser, 1994, pers. communication).

Pupillometry

The size of the pupil is a sensitive indicator of the effects of chemicals on the autonomic nervous system. Pupillometry methods have been reported for different experimental animal species: Brodie (1966), Nemec *et al* (1969) (rat, mouse); Werner (1965), Burns *et al* (1970) (rabbit); Leaders and Fortenberry (1971) (monkey).

In toxicity studies the repeated measurement of the static pupillary size in conscious dogs can be performed using a standard KOWA RC-2 fundus camera (Weisse *et al*, 1975). The camera is fixed to the laboratory bench. The height and angle of the camera are adjustable. A constant distance between the animal pupil and camera lens (64 - 67 mm) is ensured by setting the dioptre scale at -20 dioptres. In the darkened room a constant light intensity is achieved by three variable light units providing diffuse uniform illumination simulating the white wall. This evenly dispersed diffuse light is then reflected into the animal's eye. Pupils are photographed (flash intensity 5, black and white film, 50 ASA) and the pupillary diameter is measured on the developed negative. The first eye of each animal to be photographed should alternate between right and left. An interval of 90 sec. is necessary between the two photographs to allow the light reflex triggered by the flash to return to normal. This technique is sensitive enough to detect dose-effect relationships following systemic exposure to chemicals affecting the pupillary size. In rodent studies changes in the pupillary size can be measured reliably and repeatedly according to the method of Pulewka (1932). By means of a stereomicroscope (magnification 10x) equipped with an eyepiece micrometer and using constant light intensity with a constant distance between the light source and animal's eye the diameter of the pupils can be determined in conscious rats or mice (Weisse *et al*, 1971).

Tonometry

In larger experimental animals the most accurate technique to determine the intraocular pressure (IOP) is direct intrabulbar measurement (manometry). This method requires the cannulation of the anterior chamber under general anaesthesia and is therefore not suitable for conscious animals in toxicological studies (Rubin, 1992).

Since the IOP results in tension of the cornea and sclera, indirect determination of the IOP can be deduced from the resistance which the cornea exerts to opposing forces applied externally. There are three methods for estimating IOP in toxicity studies with non-rodents; digital palpation, indentation tonometry and applanation tonometry.

Digital palpation provides a crude and subjective estimation of the IOP. Two fingers are placed on the upper eyelid over the bulbus and force is applied alternately by each finger through the eyelid onto the globe. By this method an indication of either high or low IOP may be obtained although no pressure recordings are made.

Depending on the method of examination, deformation of the cornea can be produced and measured as an indentation or as applanation. Indentation tonometry is best performed using the Schiötz tonometer. This simple instrument can be used on dogs in its original form (Heywood, 1971) or as a modified electro-tonometer (Ganz *et al*, 1986). The principle is as follows: a weighted plunger slides through the centre of a concave foot plate which rests on the corneal surface and is connected to a scale which measures deflection in millimeters and this is converted into a pressure reading. For measurement the animal's head (and eyes) is positioned in a vertical axis, topical anaesthesia (0.4% novesine) is applied and 1 - 3 readings are made. In toxicological studies the Schiötz method is precise enough to allow group comparisons to establish changes in, or dose-dependency of, the IOP after exposure to chemicals despite the disadvantage of the differences in curvature of the human and canine cornea.

The measurement of the IOP by applanation tonometry is based on the determination of the force necessary to flatten a corneal area of constant size. With the hand-held Draeger applanation tonometer this is done visually with a contact slit-field prism and fluorescein or milk. The popular MacKay-Marg-tonometer uses a strain gauge that converts IOP into an electrical signal. For laboratory non-rodents both instruments have the advantage that they can be used in vertical and horizontal positions (Schiavo, 1973; Gelatt, 1981). Topical anaesthesia and manual restraint (sedation in monkeys) are necessary.

In the case of rodents, there is no suitable method for measuring the IOP in toxicity studies. In a single report continuous determination of IOP has been described in anaesthetised rats (Ohnesorge *et al,* 1968).

Examination of the Ocular Fundus

Fundus Photography

Various types of table-mounted or portable fundus cameras designed for medical fundus photography in man are available. Table-mounted instruments, in general, have superior optics, but their use for photographic recording of the fundus in laboratory non-rodents is restricted as difficulties arise when adjusting the restrained animal to the camera position. The hand-held cameras are convenient for use with animals, they deliver good-quality erect fundus photographs and are less expensive when compared to the table-mounted units. The KOWA models (e.g. RC-2, RC-3, RC-2 model 621, Genesis) are the most popular instruments in experimental ophthalmology. The KOWA RC-2 camera used for non-rodents is capable of photographing a fundus field of 15° (2 x adjustment) and 30° (1 x adjustment). The photographic fundus magnification is 2.68 x with 1 x adjustment and 5.32 x with 2 x adjustment. The focus ranges from +25 to -25 dioptres and enables the examiner to perform fundus photography in addition to making photographic records of the external eye or drainage angle with the gonioscopic prisms. Detailed exposure conditions for routine procedure should be explored individually as obtaining satisfactory fundus photographs depends on film speed, flash intensity, working distance, species-specific morphological eye characteristics or the types of pathological damage. In order to obtain good-quality fundus photographs pupillary dilation is required (in non-rodents and albino rats or mice, 1% tropicamide; in pigmented rats, 1% atropine sulphate). Due to the prominent brows and deep-set eyes, in sedated nonhuman primates the camera should be held along the horizontal plane of the eye. In the dog (cat) neutral density filters (10-30% transmittance) should be used to overcome tapetal reflection.

The KOWA RC-2 model 621 is a fundus camera specifically designed for use with small animals (rodents). Photographic magnification of 1.05 x and 2.1 x is obtained. The working distance is 2 millimeters. Dryness of the cornea can be prevented by the application of one drop of methylcellulose.

The photographic recording of the ocular fundus of animals in experimental toxicology is used to document relevant induced or spontaneous lesions of the retina, optic disc or choroid (tapetum), to demonstrate their development in the course of a study, and to give some support for the quantitative estimates. Routine photgraphic documentaion of the normal fundus before the beginning or at the end of toxicological studies should only be done in special cases.

Fluorescein Angiography

This adjunctive technique to study fundus diseases, in particular of vascular origin, can be performed in laboratory animals either by direct or indirect ophthalmoscopy or by fluorescein photography requiring special fundus cameras equipped with high speed motor drives (Newsome *et al*, 1968; Bellhorn, 1973; Gelatt, 1981). Fluorescein (10% solution) is injected intravenously and appears on the fundus in 5 - 15 seconds. As a fluorochrome, fluorescein is excited by 490 nm blue light and responds to this excitation by emitting the characteristic yellowish-green light with a maximum wavelength at 520 nm. The appropriate filters used to gain such fluorescence during fundus examination are incorporated in modern ophthalmoscopes or in the photographic unit of the fundus camera.

Under normal conditions the injected fluorescein demonstrates the retinal vessels, but does not enter the retinal tissue owing to the presence of tight junctions connecting retinal capillary endothelial cells. Even in the intact choroid, the dye leaks from the vasculature via fenestrated endothelial cells of the choriocapillaries and diffuses through the choroidal layers up to the terminal bars located between adjacent retinal pigment epithelial cells (Verhoeff's membrane). If pathological alterations of these two parts (tight junctions or terminal bars) of the so-called blood-retinal barrier occur, fluorescein leaks and stains the retinal tissue, or pooling occurs in preformed anatomical spaces (subretinal space).

The hand-held KOWA RC-2 fundus camera can be adapted for fluorescein photography by incorporating a fluorescein photographic unit with a special power supply which is capable of taking sequential photographs every 1.5-2 seconds. Black and white (Agfa-Pan APX, ASA 100), or more preferably, colour films (Kodak Ektachrome, ASA 200) can be used. For photography, mechanical restraint of the animals is insufficient: after pupil dilation (1.0% tropicamide) sedation (monkey: 0.25 ml/kg ketanest [50 mg/ml] i.m.) or short term anaesthesia (dog: sodium thiopentone 30 mg/kg, i.v., rat: sodium thiobarbital 20-30 mg/kg, i.v.) is recommended. For the dog an intravenous sodium fluorescein dosage (10% solution) of 15-20 mg/kg is suggested, whilst 7-10 mg/kg is used for the monkey. Rats receive up to 0.5 ml/animal in the tail vein. After sedation/anaesthesia a venous catheter is inserted, the camera has to be focused suitably (with the white viewing light), 1-2 pre-injection photographs are taken and fluorescein is injected rapidly. Photographic recording is initiated as the dye enters the ocular fundus. Depending on the sequence of the appearance of fluorescein the normal fluorescein angiogram consists of several phases: the choroidal (approx.

0.5-1 seconds before the retinal phases), the retinal arteriolar-capillary-venule and the recirculation. For interpretation of any abnormalities see Rubin (1974) and Gelatt (1981).

4.2.3 Specialised Methods of Clinical Eye Examination

Specialised methods are those procedures which are based on special or expensive devices, and which may require the possible help of consultants and which usually have to be performed on additional trained or sedated/anaesthetised animals (satellite groups).

Pachymetry (measurement of corneal thickness)

The intact corneal epithelium with its tight junctions in the superficial cell layers and the terminal bars in the single layered endothelium form an important semi-permeable barrier for the cornea. These structures together with the metabolic pump function of the endothelial cells help to stabilise a certain degree of stromal hydration (~78% water) and maintain the corneal thickness and transparency. Epithelial or endothelial injury or dysfunction result in corneal oedema.

Most optical methods for measuring corneal thickness in experimental ophthalmology use various image-doubling devices attached to a slit lamp microscope. The method described by Mishima and Hedbys (1968) uses two glass plates as an image splitter in front of the objective lens and a split image ocular lens (coincidence ocular) which divides the visual field horizontally into upper and lower halves. The slit lamp beam must be in an exact vertical position to the corneal surface, this position can be controlled by two small lights and by setting the slit lamp beam and the observing microscope at a 40° angle. Images of the optical section of the corneal layers occur in the upper and lower visual field of the coincidence ocular. When the image splitter is rotated, the upper half of the optical section from the cornea is displaced from the optical section in the lower half. The movable upper endothelial line and the fixed lower epithelial line have to be aligned and the displacement indicates the apparent (microscopic) corneal thickness. As the actual corneal thickness does not correspond linearly to the rotation of the image splitter, correction tables must be used.

Other optical methods for measuring corneal thickness in experimental ophthalmology have been published (Maurice and Giardini, 1951). In addition, Rubin (1992) has mentioned the possibility of measuring corneal thickness mechanically by means of specular microscopy or, with more accuracy, using an ultrasonographic device.

The routine applicability of these methods to conscious non-rodents in toxicological studies is impossible as accurate measurement of the corneal thickness depends on the exact focus of the slit lamp beam, the microscope and the alignment of the split images. This is difficult to achieve in a conscious animal. It is necessary to use sedated/anaesthetised animals; the requirement of a table-mounted slit lamp equipped with an image splitter and split image eyepiece and the employment of an experienced observer exclude pachymetry from the routine or extended ophthalmological methods.

Examination of Refraction

Apart from the rough estimation of the refractive state of the animal eye utilising the intervening convex and concave lenses of the direct ophthalmoscope, the objective evaluation of refractive errors in veterinary ophthalmology is usually done with a streak or spot retinoscope (Rubin, 1992). The measurements have to be performed in a darkened room. The pupils should be dilated. The examiner should adopt a defined distance from the animal (50 or 100 cm). A streak or spotlike beam of light is directed onto the animal's retina along the visual axis. The light is reflected through the pupil. When the ingoing light beam is moved slightly over the pupil (e.g. from left to right) the reflected light will move in the same direction (hyperopia), in the opposite direction (myopia) or does not move at all (emmetropia). The degree of refractive error is estimated by interposing convex or concave lenses directly in front of the animal's eye. When, with the aid of these lenses the movement of the reflected light is neutralized (stopped) the degree of the refractive state is determined. Afterwards correction of this refractive value has to be made by addition of -2 dioptres when the examination distance is 50 cm or -1 dioptre at a distance of 100 cm, independently of whether the refractive anomaly was myopic or hypermetropic. One has to bear in mind that animals per se have static refractive errors (e.g. dog -1.5 to -6 dioptres).

Refractive errors in dioptres have also been determined in trained dogs using a coincidence optometer (Ishikawa and Miyata, 1980) and in sedated monkeys by an automatic refractor device (Rubin, 1992).

In conscious and untrained non-rodents exact measurements are not possible owing to the spontaneous movements of the animal's eye and consequently the failure to position the ingoing light beam in the visual axis. Sedation or anaesthesia is recommended (Rubin, 1992).

Scheimpflug photography

A more refined method to record and document anterior eye segment images of laboratory rodents is, in the hands of specialists, the Scheimpflug technique (Hockwin *et al*, 1984). According to the Scheimpflug principle, the recorded images represent a non-magnified optical section with a depth of field reaching from the anterior corneal surface to the posterior lens pole. The photographed optical section (standard negative) is analysed by densitometry using a magnifying screen. The main advantage of this method, namely a substantial depth of field, ensures high reproducibility. Thus, the method can be used, in particular, first for the early detection, exact localisation and follow up-examination of cataract formation, and second for biometric examination (thickness of cornea, lens, depth of anterior chamber, curvature of cornea and lens) in toxicological studies with rodents.

Experience shows that in performing the Scheimpflug technique additional animals are not required in the course of toxicity studies (Wegener, 1994, personal communication). More methodological details are provided by Hockwin *et al* (1992a).

4.3 PATHOLOGY

Pathological examination should always be carried out on **both eyes**, optic nerves and adnexa, as the bilateral presence of findings is indicative of treatment-related effects. To avoid false negative results, in situations when only unilateral tissues are available, all abnormal findings must be considered as potentially related to treatment.

4.3.1 Standard Procedures in Pathology

The tissues should be immersed in fixative as soon as possible after killing, the eyeglobes should not be opened before fixation, nor should fixative be injected. A number of fixatives have been used of which Susa's fluid and Davidson's fixative can be recommended (Weisse, 1992; Majeed *et al*, 1987). Fixation in formalin is associated with the occurrence of artifacts which must be taken into consideration during microscopic evaluation. Prior knowledge of any ophthalmological findings is essential for the correct trimming of tissues after fixation so that the ophthalmological findings can be identified in the microscopic preparations.

The standard levels of trimming are: horizontal for monkey, pig, and rabbit, and vertical for dog, cat, and rodents (though rodents are sometimes trimmed at random). The optic nerve should always be

included in the section. One "cup" of the rodent eye and two "cups" of the eyes of other species are removed. Except for rodents, the eyes are processed without the lens. The lens of the rodent eye is kept within the bulb since it helps to retain the anatomical bulb form and the lens can be evaluated microscopically with some success. However, clinical examination of the lens is the preferred method, especially for all non-rodent species.

Following dehydration and embedding in paraffin, for routine histopathological examination, one section per eye should be prepared at the level of the optic papilla and stained with haematoxylin and eosin. Additional sections and special stains (including immunohistochemistry) should be produced when this is required for characterisation of specific findings.

Since the ocular apparatus is composed of numerous structural elements, it is essential that the topographic sites and subsites to be examined are specified in the study protocol, standard operating procedures, and pathology report. The absence of important subsites such as the lens (in rodents), the retina, or the cornea must be reported and considered in the evaluation of findings. A correlation between ophthalmologic and histopathological findings must be established in the pathology report. The degree of detail which must be specified depends on the purpose of the study and will be smaller in routine than in extended or special investigations.

Examination of the optic nerve is an essential step in the evaluation of ocular toxicity, so that the optic nerve should always be examined together with the ocular apparatus. While the retrobulbar optic fascicle is processed attached to the eye, the terminal portion of the optic tract is found in the brain as the fibre layer covering the lateral geniculate body (Hess *et al*, 1980). The optic tract is easily found in routinely prepared cross sections of the brain.

4.3.2 Extended and Specialised Procedures in Pathology

Extended pathology is required only when there is a clear and specific reason. For example, during development of ophthalmic medicaments, ocular tolerance is tested in subacute studies on rabbits, whereby the eyes with adnexa are examined in multiple step-serial sections. In toxicological safety studies, the extended procedures may include "whole mount" preparations of the retina, demonstrating this tissue with preserved topography (Schaeppi and Krinke, 1991). This technique is very tedious and should be considered only in specific situations.

Specialised procedures include fixation by vascular perfusion. Extended and specialised procedures include embedding in plastic, light microscopical examination of semi-thin and electron

microscopic examination of ultra-thin sections. Transmission electron microscopy is, for example, indispensable for the detection of induced lipidosis, damage to tapetal cells, carotenoid retinopathy, and selected corneal and conjunctival changes. However, the majority of the changes can be detected at the light-microscopic level in paraffin or semithin plastic sections. Electron microscopy may be required for characterisation of any changes and can only be conducted reasonably on a small number of appropriately selected specimens. Progression of the lesion and recovery over time is helpful for an understanding of the pathogenesis. The selected specimens for characterisation should represent early, intermediate and advanced lesions whenever possible. Appropriate control specimens should also be examined.

Scanning electron microscopy demonstrates the three-dimensionsal organisation of structures. To date, this has been especially useful for demonstration of superficial lesions in the cornea or conjunctiva.

As the extended or specialised pathological procedures for the examination of the ocular apparatus are identical to those of extended neuropathology, and since the retina forms a part of the central nervous system, it is practical to combine ocular with neuropathological examinations in protocols adapted for specific test substances.

4.4 ELECTROPHYSIOLOGY

Electrophysiological tests permit quantitative assessment of visual functions in living individuals. Non-invasive electrophysiological tests are especially suitable for medical clinical examination, where the purpose is the diagnosis and therapy resulting in an improvement of the patient's quality of life. In routine toxicological safety studies, which as a rule culminate in post mortem and histopathological examination of experimental animals, there is little need for electrophysiology because ophthalmology combined with histopathology provide satisfactory information at reasonable costs.

The quantitative nature of electrophysiological data, however, may be helpful for establishing credible safety parameters such as "maximum tolerated" or "no observable effect" doses or levels in special studies. Furthermore, sophisticated electrophysiological examination may reveal subtle functional changes which are not apparent in other tests.

Electrophysiological testing includes the electroretinogram (ERG), the electrooculogram (EOG), visual evoked cortical potentials (VECP), and the recording of eye movement. Whilst ERG and EOG diagnose retinal changes, VECP reflects the impulse conduction from the eye to the brain and may characterise damage to the visual pathway.

4.4.1 The Electroretinogram (ERG)

The electrical response elicited by light stimulation is recorded from the cornea. Rod response is received in the dark adapted (scotopic) state, cone response in the light adapted (photopic) state. For selected ERG signals both their amplitude and the implicit time (time between onset of the stimulus and the maximum response) are monitored. The characteristic ERG record consists of a-wave (attributed to the photoreceptor layer), b-wave (attributed to the Müller's and bipolar cells), and c-wave (attributed to the pigment epithelium/photoreceptor layer). Fast oscillatory potentials (attributed to the inner plexiform layer) are superimposed on the b-wave. So called "flicker ERG" is a response from cones to rapidly repeated light stimuli ("flicker trains").

Recording or rod and cone response in dogs can be carried out in dark adapted eyes using scotopically balanced red (for rods) and blue (for cones) flashes, followed by a single flash of bright white light and flicker trains of white light of various intensity (Spiess and Leber-Zürcher, 1992). For practical purposes the cone, the rod and the maximum b-wave amplitudes and implicit times are measured (Zrenner, 1992).

The ERG allows differentiation between diffuse and focal retinal lesions. A diffuse retinopathy is associated with a decrease in amplitude of the a- and b-waves, and an increase in their implicit time. Focal lesions cause a reduction of the amplitude without alteration of implicit time (Liverani and Schaeppi, 1979). Diffuse damage to photoreceptors was induced experimentally in dogs with IHBP (1-isopropyl-4-hydroxy-6-benzyl-pyrazolo (3, 4-d) pyrimidine). This condition was manifested by a decrease in amplitude of a- and b-waves and an increase in implicit time as well as the luminance needed to produce a threshold ERG. Damage to rods and cones was confirmed morphologically (Krinke and Krinke, 1979; Liverani and Schaeppi, 1979).

In comparison to man, the cone system of the dog is distinctly less sensitive. The rat has also a very limited cone function, but the rods of the rat retina appear to be as sensitive to light as in man. For ERG monitoring in rats the equipment commonly used in ophthalmology is suitable, except for light stimulation which is directly projected on the eye via an optic fibre system (Schaeppi *et al*, 1988). The ERG has been used extensively to assess the effects of organophosphorus agents, in

particular fenthion in rats. An increase in implicit times and a decrease in wave amplitudes have been reported, especially at high dose levels (Imai 1974, 1975, 1983). These findings were associated with morphological damage to the photoreceptors and retinal pigment epithelium. The retinopathy induced in rats with an anti-parasitic agent, amoscanate, was manifested by a decrease in amplitude, an increase in implicit time and an increase in ERG threshold, as well as morphologically evident atrophy of photoreceptors (Schaeppi *et al*, 1987).

As shown in Table 7, the ERG is a specialised clinical method. Its performance, as a rule, needs additional (satellite) animals, anaesthesia and special skills. In addition it is unclear how subtle functional differences detected using highly sophisticated techniques can be interpreted with regard to the assessment of human hazard.

4.4.2 The Electrooculogram (EOG)

The EOG consists of recording the standing potential between the anterior and posterior poles of the eye. This test was used to demonstrate defects in the function of retinal pigment epithelium in man (Zrenner, 1992), but the EOG is difficult to perform on laboratory animals.

4.4.3 The Visual Evoked Cortical Potential (VECP)

The cortical brain potentials evoked by visual stimulation must be discriminated from other brain potentials by means of repeated stimulation and computer averaging technique. Although the implicit time of VECP depends on the conduction velocity of the intact optic nerve, damage to the retina will be manifested by abnormal VECP as well. In amoscanate-intoxicated rats with damaged retinal photoreceptors but intact optic nerves the VECP showed a decreased amplitude and implicit time (Schaeppi *et al*, 1987). VECP is known to be sensitive to the acute effects of cholinesterase inhibitors and to show a reversible increase in implicit time.

In addition to flash evoked potentials, a "pattern reversal stimulation" is used. Pattern reversal consists of an alternating checkerboard or bar pattern of stimulation and is believed to test spatial resolution, even in rats. Pattern reversal VECP is reduced in amplitude by acute cholinesterase inhibition. Surprisingly, four days after fenthion administration to rats, the amplitude of pattern reversal VECP was increased (Boyes *et al*, 1994). In rats intoxicated with triethyltin, the pattern reversal VECP was a more sensitive indicator of retinopathy than the flash VECP (Boyes and Dyer, 1983).

Experience shows that pigmented rats are more suitable for VECP than albino rats. VECP recording is a specialised method requiring additional animals and specialised environmental conditions.

4.4.4 The Recording of Eye Movement

Abnormal eye movement can be studied in man, but is rather difficult to assess in animals. Disturbance of smooth pursuit, especially vertical movements, and increased latency for saccadic eye movement in human beings were attributed to chronic organophosphorus exposure (Ishikawa and Miyata, 1980). Such findings may reflect on nerves and muscles of the oculomotor apparatus. Nystagmus may manifest an effect on the sense of equilibrium. A technique for electronystagmography in rats has been described (Fischer *et al*, 1979).

4.5 BIOCHEMICAL METHODS

Blood clinical chemistry is used routinely to screen for early signs of major target organ damage e.g. liver and kidneys. However, biochemical methods may be employed in some instances to determine the activity or concentration of a specific analyte which is not normally measured in routine toxicology e.g. cholinesterase. These selective measurements may be made not only in blood, but also in target tissue e.g. nervous system and eye.

There are several biochemical and anatomical studies which show unequivocally that acetylcholine (ACh) is involved as a neurotransmitter in the eye. The enzymes responsible for the synthesis of ACh from choline and acetylcoenzyme A (choline acetyltransferase) and for degradation of ACh (acetylcholinesterase) are known to be present in several structures associated with the eye of a variety of vertebrate species. ACh is involved as a chemical transmitter in both the sensory and motor pathways innervating several ocular tissues (Sastry, 1985).

The major structures involved in ACh transmission will now be considered.

4.5.1 Anterior Eye Segment

Cornea

The corneal epithelium contains nerve endings of ciliary nerves and possesses relatively high concentrations of ACh. Evidence for ACh as a sensory transmitter is based upon experimental

studies involving denervation of epithelial corneal nerve terminals. Reductions in acetylcholinesterase activity of the epithelium occurred when the nerve supply was severed (Peterson *et al*, 1965).

The aqueous humour served as a primary nutrient source for the cornea and is known to contain ACh. Light-dark studies in the rabbit have pointed to a relationship between light stimulation of the eye and the ACh content of the aqueous humour (Fitzgerald and Cooper, 1971). When rabbits were kept in the dark for several hours, no ACh was found in the aqueous humour. However, upon exposure of one eye to a light source, ACh was found in the aqueous humour. The precise source of this ACh has not been established but could be the corneal epithelial cells or ciliary body.

Iris-Ciliary Body

The pupils is dilated and constricted by smooth muscle fibres which control the amount of light entering the eye. ACh has been established as a transmitter between the parasympathetic nerves and the constrictor muscles. ACh stimulates constriction of the pupil whilst anti-cholinergic substances e.g. atropine, cause dilation of the pupil. The ciliary muscle is innervated by parasympathetic nerves. ACh acts, therefore, to constrict the ciliary muscle fibres. The iris-ciliary body complex possesses both of the enzymes involved in the synthesis and degradation of ACh (Sastry, 1985).

4.5.2 Retina

Of all the ocular tissues, the retina provides the most extensive and unequivocal evidence that ACh is involved in sensory transmission. Several reviews are available to confirm this assertion (Masland and Tauchi, 1986; Hutchins, 1987). Retina from a number of species has been shown to contain the enzymes responsible for the synthesis and degradation of ACh. Histochemical methods have localised AChE activity to the inner retina in almost all animal species examined (Francis, 1953, Graham, 1974). Overall the properties of the enzyme (characterised in several laboratory species) are quite similar to that reported for mammalian brain AChE (Hutchins, 1987). Choline acetyltransferase activity has been detected in homogenates of cat, rabbit, cow and human retina (Mindel and Mittag, 1976). Furthermore, choline acetyltransferase activity appears to correlate directly with the number of amacrine cell synapses with few exceptions (Ross and McDougal, 1976), providing evidence, by implication, that ACh is transmitter at pre-synaptic neurones of bipolar and/or amacrine cells. In addition, both biochemical and anatomical studies of the high affinity

uptake of the precursor choline into retinal tissue have demonstrated the synthesis of ACh from radiolabelled-choline (Masland and Mills, 1979; 1980).

A comparison of cholinergic markers in the retina of various species can be found in Sastry (1985). The release of ACh in response to photic stimulation has been demonstrated *in vivo* in rabbits (Neal and Massey, 1980) and *in vitro* (Masland and Livingstone, 1976). Retinal neurones depolarised by light are cholinergic. In mammalian retinas, cholinergic neurones are predominantly amacrine cells and displaced amacrine cells localised to the ganglion cell layer (Masland *et al*, 1984). Transmission of nerve impulses in the retina is not confined to ACh and there is evidence that several other transmitter agents (known to be involved in the nervous system are also involved (Massey and Redburn, 1987).

4.5.3 Cholinesterases

Two principal types of cholinesterase enzyme have been identified:

- acetylcholinesterase (AChE; EC 3.1.1.7); the enzyme is characterised by its preferential affinity for ACh as a substrate; it is also referred to as 'true' cholinesterase; AChE is known to be the predominant enzyme in the nervous system (including ocular tissues) and erythrocytes;

- cholinesterase (EC 3.1.1.8) is found in several tissues including blood plasma and liver, it exhibits heterogeneity and shows less substrate specificity than AChE; it is also referred to a 'pseudo' - or 'non-specific' cholinesterase.

Both enzymes are known targets for certain compounds, including organophosphorus and carbamate esters, which are widely used as insecticides.

Several biochemical and histochemical procedures have evolved for the measurement of acetyl cholinesterase activity (Whittaker, 1986). The electrometric pH method (Michel, 1949) was for many years the method of choice. It has now been superseded by a colorimetric method (Ellman *et al*, 1961) and its modifications for different species (Voss and Schasse, 1970; Pickering and Pickering, 1971). The colorimetric method offers significant advantages in speed, sensitivity and reliability. However, it is subject to interference from endogenous sulphydryl materials in tissue and from haemoglobin. The other major biochemical method has involved the use of radiolabelled ACh (Johnson and Russel, 1975). Although the radiochemical assay principle is rapid and relatively

simple, this approach has diminished in popularity due largely to the problems of disposal of radioactive waste. Apart from biochemical methods of assay, a quantitative histochemical method has been established for the measurement of AChE activity in brain tissue (Biegon and Wolff, 1986).

There are significant pitfalls associated with the measurement of cholinesterase activity. The inhibited enzyme may undergo spontaneous reactivation relatively rapidly, as can occur with several carbamate esters. If the incubation time for the assay is significant in relation to this rate of reactivation, then the degree of inhibition may be underestimated. Conversely, further inhibition of the enzyme may occur after tissue sampling due to the presence of free inhibitor. Thus, if the rate of inhibition of the enzyme by free inhibitor exceeds the rate of spontaneous reactivation, then the degree of inhibition may be overestimated. It is essential, therefore, in this assay to ensure that time which elapses between tissue sampling and assay (including the incubation period) is kept to the minimum.

In toxicology studies where organophosphorus or carbamate esters are to be evaluated, it has been customary to use the dog and the rat as the preferred species for cholinesterase measurements in blood and target tissues. In all examples where cholinesterase inhibition was the critical effect, the dog was as sensitive or more sensitive than the rat (Appelman and Feron, 1986). However, the route of intake of the compound and differences in eating habits between the various species, including man, will influence the assessment of species sensitivity.

The measurement of AChE activity in target tissues e.g. brain will continue to be a requirement of regulatory studies when testing for the effects of anticholinesterase agents. Traditionally for brain the whole or part organ has been dissected and homogenised in a buffered medium to provide a tissue suitable for assay. However, measurements made on whole tissue do not take account of the variations in activity which are known to exist in different anatomical regions (Mesulam *et al*, 1986). Significant changes in the activity in sensitive regions may be hidden by relatively small but subtle effects in regions of high AChE activity.

Retinal AChE activity has been shown to be approximately twice as high as that in the cerebellum and cerebrum of Wistar rats (Imai, 1974). In Beagle dogs, the highest AChE activity was found in extra-ocular muscle (Hikita *et al*, 1973). The enzyme in ocular tissue of rats has been confirmed as mainly AChE rather than pseudocholinesterase (Imai, 1974).

Measurements of AChE activity in blood, brain and ocular tissues have been compared following administration of certain organophosphorus agents. The dose-response relationship between AChE activity of the retina and cerebellum in rats after a single dose of fenthion showed a similar pattern with both activities reduced at doses of 0.5 mg/kg or greater (Imai, 1974); however, these findings were not confirmed in a study using the same concentration range of fenthion in a different strain of rat (Boyes *et al*, 1994). The reason for this apparent difference in sensitivity is unclear. Nevertheless, the latter study did substantiate the finding that the depression of AChE activity was approximately equivalent in brain and retina (Boyes *et al*, 1994). A comparison of the rate of inhibition and recovery of retinal AChE activity and plasma pseudocholinesterase activity following dosing of fenthion to rats showed a similar pattern and degree of inhibition although recovery to normal activity was slightly longer for the retinal enzyme (Imai, 1975). In Beagle dogs given ethylthiometon, AChE activities in several organs including extra-ocular muscle, retina, uvea, mid-brain and spinal cord were reduced with increasing dose whilst serum pseudocholinesterase activity was unaffected (Hikita *et al*, 1973). Several enzyme activities including AChE were measured in ocular tissues of dogs given fenitrothion; AChE activity was reduced in the ciliary body but not in the lens when compared to control dogs (Ishikawa and Miyata, 1980).

Until scientifically sound and validated data are generated which demonstrate the relevance of measuring acetylcholinesterase activity in discrete regions of the ocular system, measurements of acetylcholinesterase activity in brain appear to offer a reasonable estimate of the extent of absorption and effect in the central nervous and ocular system. The use of only a portion of brain tissue for biochemical measurements offers a more pragmatic approach as the remaining brain tissue can still be used for histopathology. In contrast, when one eye is taken for biochemical measurements, it is lost for histopathology.

SECTION 5. GUIDELINES FOR OCULAR TOXICITY TESTING

Regulatory toxicity studies in this monograph refer to the toxicity test guidelines of OECD (1981a,b,c, 1987), EEC (1983, 1987a,b, 1988, 1992), US-EPA FIFRA (1984), US-EPA TSCA (1985), US-FDA (1982), Japan-MAFF (1985) and Japan-MHW (1990). Relevant information regarding the potential for ocular toxicity can be obtained from acute (single dose), subacute, subchronic, chronic and developmental studies. Screening for ocular irritancy has been addressed earlier (ECETOC, 1988) and is not covered here.

The published toxicity test guidelines cover the use of agrochemicals, food additives, industrial chemicals and pharmaceuticals. There are, however, no prescribed guidelines issued by the FDA for pharmaceuticals (where a case by case basis is followed) or by the EEC for agrochemicals. In contrast, the US-FDA is the only regulatory authority to issue prescribed guidelines for food additives (US-FDA, 1982) and these are currently undergoing revision (US-FDA, 1993).

For repeat dose studies, several published guidelines make specific reference to the need for ophthalmological and histopathological examination of the eye in both rodent and non-rodent species. All subchronic studies include guidance on the numbers of animals and the frequency of ophthalmological examination. For chronic studies involving industrial chemicals, there is a requirement for ophthalmological examination by the US-EPA but not by the EEC and OECD. For both subchronic and chronic studies, histopathological examination of the eye is usually required for all non-rodents. Histopathological examination of the eyes of all control and high dose rodent groups is usually required by most regulatory authorities; however, some only require histopathology if indicated by signs of toxicity or if the eye is a target organ, e.g. US-EPA. None of the guidelines contains any specific recommendations as to how the examination should be performed. It is our view that in chronic toxicity studies the primary histopathological examination should be done for high dose and control groups and all decedents only.

Several OECD test guidelines are under review currently e.g. "Number 407; Repeat dose 28 day oral toxicity study". The revised guideline makes no specific reference to screening for ocular toxicity by either ophthalmological or histopathological examination. The eye would only be processed and examined if macroscopically abnormal.

Current test guidelines on teratogenicity issued by all regulatory authorities have the provision to look for external abnormalities including eye defects e.g. anophthalmia, microphthalmia.

An overview of guideline recommendations for ophthalmology and histopathology for agrochemicals (US-EPA (FIFRA), 1984; Japan-MHW, 1985), food additives (US-FDA, 1982, 1993), industrial chemicals (US-EPA (TSCA) 1985; OECD, 1981a,b,c, 1987), and pharmaceuticals (Japan-MHW, 1990; EEC, 1983,1987a,b) is presented (Appendix A, Table A1 to A4). Subsequently a more detailed breakdown of the study requirements is given (Appendix A, Table A5).

The guidelines, in principle, cover adequately the testing requirements in toxicity studies, though some paragraphs appear ambiguous (see Appendix A). Harmonisation of the guidelines by the various agencies (as for example by OECD) would be of value.

SECTION 6. STRATEGY IN TESTING FOR OCULOTOXICITY

The following practical testing strategy is recommended for the identification, characterisation and assessment of oculotoxic effects in animal studies. It should apply equally to the testing of agrochemicals, food additives, industrial chemicals and pharmaceuticals, although testing of ophthalmic medicaments requires a specific approach. It involves the extension of existing guidelines to allow for the conduct of studies in such a way as to identify potential oculotoxicity. The probability of detecting ocular toxicity is enhanced when both rodent and non-rodent species are used.

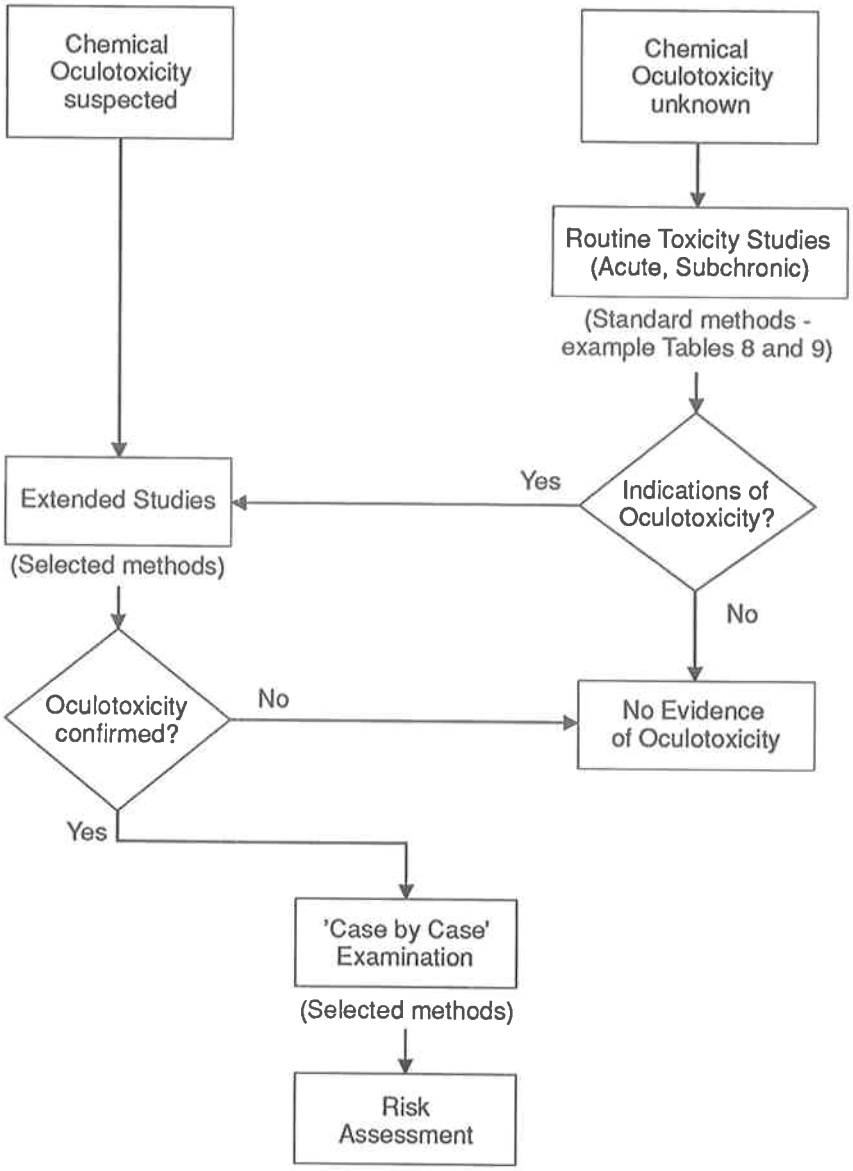
The testing for oculotoxicity should be performed in a stepwise manner (as depicted in Figure 3) starting with routine toxicity studies. It is recommended, in addition to requirements specified in some regulatory Guidelines, that histopathology of the eye should be incorporated into those studies where the design includes histopathological examination of the nervous system, as a means of assessing the neurotoxic potential of a chemical. Furthermore, in routine studies where ophthalmology and histological examination are the main procedures used, the ocular adnexa such as the lacrimal and Harderian glands should be preserved for histopathological examination in case of findings in the eye and the optic nerve, since the changes observed in the ocular adnexa may contribute to their interpretation.

When the potential for oculotoxicity has been identified with a compound, it may be necessary to extend the existing techniques of ophthalmology and histology by performing additional relevant measurements and by using more extended or specialised techniques. Examples are provided in Tables 8 and 9.

Extended or specialised methods, when considered necessary, should be tailored to characterise the suspected oculotoxicity of particular chemical agents. "Case by case" decisions about selection of additional procedures and "custom made" design of special studies, including the desirability to demonstrate the reversibility of any observed changes are recommended.

There is an insufficient scientific database for defining detailed testing rules for specific chemical or biological classes of chemicals. This is evident from the recent proposed protocols for the determination of potential ocular effects of OP pesticides (Hamernik, 1994), where a variety of examinations is suggested, but simultaneously identified as "optional" (cholinesterase assay in various tissues) or "open for discussion" (ERG, VECP).

Figure 3 Order of Activities for Testing Oculotoxicity



Whilst some of the specialised animal models designed to replicate human ocular disease provide an insight into its pathogenesis, the correlation between oculotoxicity in man and animal safety evaluation studies is generally poor, since animal studies may show effects irrelevant for human beings. Examples of the methods which have been used to characterise the ocular effects of some OPs in dogs and rats are shown in Tables 10 and 11.

Table 8 Rat: example of methods for standard, extended and specialised examination.

Methods	Standard	Extended	Specialised
CLINICAL			
- slit lamp biomicroscopy	+	+	
- indirect ophthalmoscopy	+		
- slit lamp photography		+	
- pupillometry		+	
- fundus photography		+	
- fluorescein angiography		+	
- Scheimpflug photography/biometry			+
- Schirmer tear test			+
- electroretinography (ERG)			+
- visual evoked cortical potential (VECP)			+
PATHOLOGY			
- light microscopy of the eye and the optic nerve, HE stain, and optional special stains	+	+	+
- light microscopy of the ocular adnexa		+	+
- perfusion fixation			+
- electron microscopy on selected specimens		+	+

Taken together, the various animal studies performed for studying alleged chronic human effects have yielded vast quantities of data demonstrating a spectrum of functional, biochemical and morphological effects of OPs on the animal ocular system. A review of the reported findings shows that most changes are detected in routine studies using ophthalmology and histopathology. It is only on rare occasions that more extended or specialised techniques are needed to provide further clarification. These include for example, measurement of corneal curvature and corneal astigmatism by ophthalmometry, measurement of corneal thickness by pachymetry, electroretinography for testing of retinal abnormalities and visual evoked cortical potentials for testing visual pathways. The main advantages of electrophysiological techniques are that they provide an assessment of functional integrity and can be used in the in-life phase of studies. However, non-invasive techniques are not essential in toxicity studies where ophthalmology and histology provide a sensitive and reliable assessment of the potential for oculotoxicity.

For selected compounds, it may be necessary to define the toxicokinetics and mechanism of toxic action in order to place the observations into context for human exposure and to provide a basis for a risk assessment to be made. Moreover, the predictive value of animal findings for man must not be exaggerated, since the human visual system is able to perform functions which have developed differently from those of animals and therefore standard testing is inappropriate.

Table 9 Dog: example of methods for routine, extended and specialised examination.

Methods	Standard	Extended	Specialised
CLINICAL			
- examination with focal illumination	+		
- direct/indirect ophthalmoscopy	+		
- slit lamp biomicroscopy		+	
- slit lamp photography		+	
- external ophthalmic stains		+	
- Schirmer tear test		+	
- esthesiometry		+	
- pupillometry		+	
- tonometry		+	
- fundus photography		+	
- fluorescein angiography		+	
- pachymetry			+
- examination of refraction			+
- electroretinography (ERG)			+
PATHOLOGY			
- light microscopy of the eye and the optic nerve, HE stain, and optional special stains	+	+	+
- light microscopy of the ocular adnexa		+	+
- perfusion fixation			+
- electron microscopy on selected specimens		+	+

Table 10 Characteristic findings and methods used for effects reported with selected organophosphorus compounds in rats

Structures and Effects		Methods	
Structure	Effect	Clinical	Pathology
cornea	dystrophy, neo-vascularisation	biomicroscopy	light microscopy
lens	cataract	biomicroscopy	light microscopy
retina	degeneration, ERG abnormal, retinal vascular constriction	ophthalmoscopy, ERG	light microscopy
optic nerve	reduced number of axons	VECP	light microscopy (semi-thin section)

Table 11 Characteristic findings and methods used for effects reported with selected organophosphorus compounds in dogs

Structures and Effects		Methods	
Structure	Effect	Clinical	Pathology
cornea	astigmatism	ophthalmometry of corneal curvature	
	myopia	(refractive error) retinoscopy (refractive error) ophthalmometry pachymetry (corneal thickness)	
extraocular and ciliary muscle	degeneration, membranous inclusions		light microscopy
retina	degeneration	ophthalmoscopy, ERG	light microscopy
optic nerve	reduced number of axons	VECP	light microscopy (semi-thin section)
intraocular pressure	elevation	Schiötz tonometry	(light microscopy)

Ocular toxicity in man following long term exposure to chemical substances is a matter of particular concern. Changes observed in chronic rodent studies reflect for the most part species-specific senile changes which are considered not to be predictive of long term effects to the human ocular system. Furthermore, the interpretation of ocular findings can be confounded by physiological changes. For instance, when OPs are tested, there is sustained excitation of cholinergic systems which can result in secondary effects such as a failure of lacrimation. Thus, carefully designed studies of shorter duration appear more appropriate for studying the possible effects in man.

In summary, the strategy described above provides a structured approach to the identification and characterisation of potential oculotoxic compounds but also retains flexibility to develop studies of an appropriate design which incorporate only the most relevant techniques.

APPENDIX A. GENERAL GUIDELINES FOR OCULAR TOXICITY TESTING.

Table A1 Agrochemicals: Selected ocular toxicity testing requirements from systemic toxicity guidelines as required in the United States (US-EPA (FIFRA), 1984) and Japan (J-MAFF, 1985). (HD = high dose group, C = control group)

	(US-EPA (FIFRA), 1984)	(J-MAFF,1985)
single dose toxicity study	rodents (non-rodents): Ophthalmology/Histopathology: not mentioned	rodents (2nd mammalian species): Ophthalmology/Histopathology: not mentioned
repeated dose toxicity study	**90 days** rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity or if eye is target organ (in inhalation studies: the eyes of all HD and C animals, routinely) non-rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity or if eye is target organ **12 months or longer** rodents: Ophthalmology: pre-dose and at termination at least 10 m/10f each dose level Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: the eyes of all animals	**90 days** rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: the eyes of all animals **12 months (non-rodents) or 18/24 months (rodents)** rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: the eyes of all animals

	(US-EPA (FIFRA), 1984)	(J-MAFF,1985)
combined chronic toxicity and carcinogenicity study	**12/18 or 24 months** rodents: Ophthalmology: pre-dose and at termination at least 10m/10f each dose level Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**12/18 or 24 months** rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently
carcinogenicity study	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently

Table A2 Food Additives: Recommended ophthalmological and histopathological examinations of the eyes in systemic toxicity studies as required in the United States (US-FDA, 1982, 1993. (HD = high dose group, C = control group)

	(US-FDA, 1982)	(US-FDA, 1993) in draft form
single dose toxicity study	rodents: Ophthalmology/Histopathology: not mentioned	rodents: Ophthalmology/Histopathology: not mentioned
repeated dose toxicity study	**4 weeks or less** rodents: Ophthalmology: pre-dose and at termination at least 5 m/5 f HD and C animals Histopathology: not mentioned non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: not mentioned	**14/28 days** rodents: Ophthalmology: pre-dose and at termination on designated animals Histopathology: the eyes of all animals non-rodents: Ophthalmology: pre-dose and at termination on designated animals Histopathology: not mentioned
repeated dose toxicity study	**90 days** rodents: Ophthalmology: pre-dose and at termination at least 10 m/10 f HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: the eyes of all animals	**90 days** rodents: Ophthalmology: pre-dose and at termination on designated animals Histopathology: the eyes of all animals non-rodents: Ophthalmology: pre-dose and at termination on designated animals Histopathology: the eyes of all animals
repeated dose toxicity study	**12 months** or longer rodents: Ophthalmology: pre-dose, every 3 months thereafter and at termination all HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently non-rodents: Ophthalmology: pre-dose, every 3 months thereafter and at termination all animals Histopathology: the eyes of all animals	**12 months** non-rodents: Ophthalmology: pre-dose, every 3 months therafter and at termination on designated animals Histopathology: the eyes of all animals

	(US-FDA, 1982)	(US-FDA, 1993) in draft form
combined chronic toxicity and carcinogenicity study	**12/24 months** rodents: Ophthalmology: pre-dose, every 3 months thereafter and at termination all HD and C animals Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**12/24 months** rodents: Ophthalmology: pre-dose, every 3 months therafter and at termination on designated animals Histopathology: the eyes of all animals
carcinogenicity study	**24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all animals

Table A3 Industrial Chemicals: Recommended ophthalmological and histopathological examinations of the eyes in systemic toxicity studies as required in the United States (US-EPA [TSCA], 1985), the European Commision (EEC, 1988, 1992) and as recommended by OECD (OECD, 1981,1987). (HD = high dose group, C = control group)

	(US-EPA (TSCA), 1985)	(OECD, 1981, 1987)	(EEC, 1988, 1992)
single dose toxicity study	rodents and non-rodents: Ophthalmology/Histopathology: not mentioned	rodents and non-rodents: Ophthalmology/Histopathology: not mentioned **28/14 days** Ophthalmology/Histopathology: not mentioned	rodents or rabbits: Ophthalmology/Histopathology: not mentioned **28 days** rodents: Ophthalmology/Histopathology: not mentioned
repeated dose toxicity study	**3 months** rodents or rabbits: Ophthalmology: pre-dose and at termination 5m/5f each group Histopathology: if indicated by signs of toxicity non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: if indicated by signs of toxicity **12 months or longer** rodents: Ophthalmology: pre-dose and at termination 10m/10f each group Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: pre-dose and at termination all animals Histopathology: the eyes of all animals	**90 days** rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity non-rodents: Ophthalmology: pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity **12 months or longer** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died /killed intercurrently	**90 days** rodents or rabbits: Ophthalmology: Pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity or if eye is target organ non-rodents: Ophthalmology: Pre-dose and at termination at least HD and C animals Histopathology: if indicated by signs of toxicity or if eye is target organ **12 months or longer** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently non-rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently

	(US-EPA (TSCA), 1985)	(OECD, 1981, 1987)	(EEC, 1988, 1992)
combined chronic toxicity and carcino-genicity study	**12/24 months** rodents: Ophthalmology: pre-dose and at termination 10m/10f each dose level Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**12/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**12/18 or 24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently
carcino-genicity study	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/sacrificed intercurrently

Table A4 Pharmaceuticals: Recommended ophthalmological and histopathological examinations of the eyes in systemic toxicity studies as required by the European Commission (EEC, 1983, 1987) and Japan (J-MHW, 1990). (HD = high dose group, C = control group)

	(J-MHW, 1990)	(EEC, 1983,1987)
single dose toxicity study	rodents and non-rodents: Ophthalmology/Histopathology: not mentioned	rodents and non-rodents: Ophthalmology/Histopathology: not mentioned
repeated dose toxicity study	**1-12 months** rodents: Ophthalmology: at least once during administration a fixed number of animals of each group Histopathology: the eyes of all HD an C animals and all animals which died/killed intercurrently non-rodents: Ophthalmology: pre-dose and at least once during administration of all animals Histopathology: the eyes of all animals	**2 weeks - 6 months** rodents: Ophthalmology: pre-dose and at termination limited number from each group Histopathology: the eyes of all HD and C animals non-rodents: Ophthalmology: pre-dose and at termination, not otherwise specified Histopathology: the eyes of all animals
carcinogenicity study	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently	**18/24 months** rodents: Ophthalmology: not mentioned Histopathology: the eyes of all HD and C animals and all animals which died/killed intercurrently

Table A5 Toxicology Testing Requirements with the empasis on the evaluation of ocular toxicity

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
EPA 1984 PAG (FIFRA)	Subchronic oral (82-1)	Rat and dog preferred Rat < 8 weeks of age 10/sex/group Dog 4-9 months of age 4/sex/group min. 3 levels & control	Pre-dose & termination All controls & top dose If effects seen at top dose other groups to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic dermal (82-3)	Rat, rabbit or guinea pig (albino rabbit preferred) young adults 10/sex/group min. 3 levels & control	**Guidelines ambiguous** All animals/sex/group? All controls & top dose If effects seen at top dose other groups to be examined? **(probably the latter).**	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic inhalation (82-4)	Rat preferred young adults 10/sex/group min. 3 levels & control	Pre-dose & termination 10 animals/sex/group, controls & top dose.	All control & top dose animals. Other groups if signs of toxicity or pathological changes present.
	Chronic toxicity (83-1)	Rat and dog preferred Rat 20/sex/group < 8 weeks of age Dog 4/sex/group 4-9 months of age min. 3 levels & control	Pre-dose & termination All groups of non-rodents. At least 10/sex/group of rodents, if effects seen all to be examined.	All groups of non-rodents. All control & top dose for rodents, other groups if signs of toxicity or pathological changes present.
	Combined chronic toxicity/ carcinogenicity (83-5)	Rat preferred < 8 weeks of age 50/sex/group min. 3 levels & control	Pre-dose & termination. All groups of non-rodents. At least 10/sex/group of rodents, if effects seen all to be examined.	Only if gross lesion present or if eye is a target organ.
FDA (1982) Toxicological principles for the safety assessment of direct food addit. & colour addit. used in food	Short-term continuous exposure oral toxicity (1 month or less)	Rats and dogs Rat <6 weeks of age 10/sex/group Dog 4-6 months of age 4/sex/group min. 3 levels & control	Pre-dose & termination. All dogs. At least 5 rats/sex/group for top dose and controls, if changes seen all other animals to be examined.	Only if gross lesions present.

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
FDA (1982) Toxicological principles for the safety assessment of direct food addit. & colour addit. used in food (cont.)	Subchronic oral	Rats and dogs Rat <6 weeks of age 20/sex/group Dog 4-6 months of age 4/sex/group min. 3 levels & control	Pre-dose & termination. All dogs. At least 10 rats/sex/group for top dose and controls, if changes seen all other animals to be examined.	All dogs. All control & top dose rats plus any rat killed or found dead.
	Long-term rodent (at least 1 year)	Rat approx 6 weeks of age 20/sex/group min. 3 levels & control	At start, every 3 months during study and termination. All top dose & controls, if changes seen all other animals to be examined.	All control & top dose plus any killed or found dead, if equivocal results or abnormality seen all other animals to be examined.
	Long-term dog (1 year)	4-6 months of age 4/sex/group min. 3 levels & control	All animals at start, every 3 months during study & termination.	All animals.
	Combined chronic oral/ carcinogenicity in rodent	Rat preferred < 6 weeks of age 50/sex/group min. 3 levels & control	At start, every 3 months & termination. All top dose & controls if changes seen all other animals to be examined.	All control & top dose plus any killed, found dead or with lesions. If equivocal results or abnormality seen all other animals to be examined.
Japanese Agricultural Laws & Regulations (1984)	Subchronic oral (90 day)	Rat and dog preferred Rat <8 weeks of age 10/sex/group Dog 4-6 months of age 4/sex/group min. 3 levels & control	Pre-dose & termination. All dogs. At least top dose & control rats, if changes seen all other animals to be examined.	All dogs. All control & top dose rats, any rat killed or found dead during study & if gross lesion present. If effects seen all other animals to be examined.
	Subchronic dermal (21 day)	Rat, rabbit or guinea pig adult 5/sex/group min. 3 levels & control	Pre-dose & termination. At least top dose & control, if changes seen all other animals to be examined.	Only if eye is a target organ.
	Subchronic inhalation (90 day)	Rat preferred young adult 10/sex/group min. 3 levels & control	Pre-dose & termination. At least top dose & control, if changes seen all other animals to be examined.	All control & top dose plus any found dead, killed during study or with gross lesions. Other groups to be examined if effects seen in top group.

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
Japanese Agricultural Laws & Regulations (1984) (cont.)	Chronic toxicity	Rat & dog preferred Rat <6 weeks of age 20/sex/group Dog 4-6 months of age 4/sex/group min. 3 levels & control	Pre-dose & termination. All dogs. At least top dose & control for rats. If changes seen all other animals to be examined.	All non-rodents. All control & top dose rats plus any found dead, killed during the study or with gross lesions. Other groups to be examined if eye target organ or if effects seen at top dose.
	Combined chronic toxicity/ oncogenicity	Rat <8 weeks of age 50/sex/group min. 3 levels & control	Pre-dose & termination. At least top dose and controls. If changes seen all other animals to be examined.	All control & top dose plus any found dead, killed during the study or with gross lesions. Other groups to be examined if eye target organ or if effects seen at top dose.
CPMP Guidelines on Medicinal Products for Human Use. Rules Vol. 3 Annex to Directive 75/318/EEC	Repeated dose toxicity (2 weeks - 6 months)	2 different species incl. 1 non-rodent (species not specified) Equal numbers of each sex/ group (group sizes not specified) min. 3 levels & control	Pre-dose & termination. All non-rodents. A limited number of rodents at each dose level (numbers not specified).	All non-rodents. All rodents from control & top dose, other groups only if pathological changes seen at autopsy.
Guidelines for toxicity study of drugs. Pharmaceuticals Affairs Bureau, Ministry of Health & Welfare, Japan, 1990	Repeated Dose Toxicity (1, 3, 6 or 12 months)	2 different species including 1 non-rodent - mature animals Rodents - 10/sex/group Non-rodents - 3/sex/ grp min. 3 levels & controls	All non-rodents predose and at least once during period of administration. A fixed number of rodents from each group at least once during period of administration.	All non-rodents. All top dose & controls rodents
OECD	Subchronic oral toxicity - rodent (408)	rat preferred < 8 weeks of age 10/sex/group min. 3 levels & controls	Pre-dose & termination. All top dose and controls. If changes in eye detected, all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic oral toxicity - non-rodent (409)	dog preferred 4-9 months of age 4/sex/group min. 3 levels & controls	Pre-dose & termination. All top dose & controls. If changes in eye detected, all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic dermal toxicity (411)	rat, rabbit or guinea pig young adults 10/sex/group min. 3 levels & controls	Pre-dose & termination. All top dose & controls. If changes in eye detected, all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
OECD (cont.)	Subchronic inhalation toxicity (413)	Usually rat young adult 10/sex/group min. 3 levels & controls	Pre-dose & termination All top dose & controls if changes in eye detected all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Chronic toxicity (452)	Rodent and dog or primate young animals, shortly after weaning Rodents - 20/sex/group Non-rodents - 4/sex/group min. 3 levels & controls	NOT specified. However does state "... clinical signs, including ... ocular changes should be recorded for all animals".	All control & top dose plus any found dead or killed during the study if abnormalities seen at top dose all other animals to be examined.
	Combined chronic toxicity/ carcinogenicity (453)	Usually rat <6 weeks of age 50/sex/group min. 3 levels & controls	NOT specified. However does state "... clinical signs, including ... ocular changes should be recorded for all animals".	All control & top dose plus any found dead or killed during the study if abnormalities seen at top dose all other animals to be examined.
Commission Directive 87/302/EEC EC Annex V Official Journal of the EC	Non-rodent subchronic oral toxicity	Dog preferred 4-6 months of age 4/sex/group min. 3 levels & controls	Pre-dose & termination. All top dose & controls if ocular changes seen all remaining animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Chronic toxicity	Rat preferred soon after weaning Rodent - 20/sex/group Non-rodent- 4/sex/group min. 3 levels & control	Not specified. **Guidelines are ambiguous**. They state "... clinical signs ... including ocular changes ... to be recorded" (but state that report should include ophthalmological findings).	All control & top dose animals plus any killed or found dead during study.
	Combined chronic toxicity/ carcinogenicity	Rat - young animals main study 50/sex/group, satellite groups 20/sex/group satellite controls 10/sex min. 3 levels & controls & satellite test and control groups for assessment of chronic toxicity.	Not specified. **Guidelines are ambiguous.** They state "...clinical signs ... including ocular changes ... to be recorded" (but state that report should include ophthalmological findings).	All satellite top dose and satellite control animals. If effects seen all remaining satellite animals & all animals from main study to be examined.

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
Commission Directive 87/302/EEC EC Annex V Official Journal of the EC (cont.)	Subchronic oral toxicity (90 day repeated oral dose using rodent species)	Rat preferred <8 weks of age 10/sex/group min. 3 levels & controls	Pre-dose & termination. All controls & top dose if effects seen at top dose all other animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic dermal toxicity	Rat, rabbit or guinea-pig young adult, 10/sex/group min. 3 levels & controls	Pre-dose & termination. All controls & top dose. If effects seen at top dose all other animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic inhalation toxicity	Rat preferred young animals, 10/sex/group min. 3 levels & controls	Pre-dose & termination. All controls & top dose. If effects seen at top dose all other animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
EPA (TSCA)	Subchronic oral 798.2650	Rat and dog preferred Rat <8 weeks of age, 10/sex/group Dog 4-6 months of age, 4/sex/group min. 3 levels & controls	Pre-dose & termination. All controls & top dose. If effects seen at top dose other groups to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic dermal 798.2250	Rat, rabbit or guinea pig (rabbit preferred) young adults, 10/sex/group min. 3 levels & control	Pre-dose & termination. At least 5/sex/group. If effects seen all all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Subchronic inhalation 798.2450	Rat preferred Rat young adults, 10/sex/group Non-rodent young adult, 4/sex/group min. 3 levels & controls	Pre-dose & termination. At least 5/sex/group. If effects seen all animals to be examined.	Only if indicated by signs of toxicity or if eye is a target organ.
	Chronic toxicity 798.3260	Rat and dog preferred Rat <8 weeks of age, 20/sex/group Dog 4-6 months of age 4/sex/group min. 3 levels & controls	Pre-dose & termination. All non-rodents. At least 10 rodents/sex/group.	All non-rodents. All top dose & control rodents plus any that died or were killed during the study. All animals if eye is a target organ.

REGULATORY BODY	STUDY/GUIDELINE REF.	SPECIES, AGE, GROUP SIZE, DOSE LEVELS	OPHTHALMOLOGICAL EXAMINATION	EYE HISTOPATHOLOGY
EPA (TSCA) cont.	Combined chronic toxicity/ oncogenicity 798.3320	Rat <8 weeks of age 50/sex/group for main study and 20/sex/group for satellite groups (for assessment of chronic toxicity) Min. 3 levels & controls plus at least & satellite dose group & satellite control group.	Pre-dose & termination. At least 10/sex/group. If effects seen all animals to be examined.	All control & top dose animals plus any that died or were killed during the study.

GLOSSARY

ACETYLCHOLINESTERASE (AChE):

Enzyme which is found in several tissues including the ocular system and which is responsible for the inactivation of the neurotransmitter, acetylcholine.

ANGIOGRAPHY:

Visualisation of vessels by the use of fluorescent or radioopaque substances.

ANOPHTHALMIA:

Developmental defect characterised by absence of the eye.

ANTERIOR CHAMBER

cf Figure 1.

AQUEOUS HUMOUR:

Fluid occupying the anterior and posterior chamber of the eye and diffusing out of the eye into the blood.

APHAKIA:

Developmental defect characterised by the absence of the lens.

ASTIGMATISM:

Optical condition in which the refractive power is not uniform in all meridians.

ATROPHY:

Acquired diminution in size.

a-WAVE:

Initial wave of electroretinogram.

AXONOPATHY:

Axonal disease.

b-WAVE:

Positive wave following a-wave of electroretinogram.

BLEPHARITIS:

Inflammation of the eyelids.

BLEPHAROSPASM:

Spasm within the circular muscles causing more or less complete closure of the eyelids.

BOWMAN'S MEMBRANE:

Specialised layer of corneal stroma beneath outer stratified epithelium.

BRUCH'S MEMBRANE:

Underlying membrane separating the cells of the retina from the choroid (cf figs 1 and 2).

BULBAR CONJUNCTIVA:

Mucous membrane covering anterior part of sclera.

CATARACT:

Indicates loss of transparency of the lens, or opacity.

CENTRAL AREA:

Seen in species such as dogs and corresponds to macula lutea of primates ie a retinal concentration of cones.

CENTRAL RETINAL ARTERY:

Major artery supplying the retina in some species.

CHOLINE ACETYLTRANSFERASE:

Enzyme which is found in several tissues including the ocular system and which is involved in the synthesis of the neurotransmitter, acetylcholine.

CHOROID:

Pigmented (except albinos) vascular coat of the eye extending from the ora serrata to the optic nerve.

CHROMODACRYORRHOEA:

Shedding of tears containing reddish (porphyrin) secretion from Harderian gland.

CILIARY BODY:

Supports the lens enabling accommodation by use of ciliary muscle.

CILIARY PROCESSES:

Projections from ciliary body.

CILIORETINAL ARTERIES:

Posterior ciliary arteries connecting choroidal and retinal vasculature.

COLOBOMATOUS DEFECT:

Developmental defect of the ocular tissue usually associated with the failure of foetal tissue to close.

CONE-MEDIATED:

Response carried out by cone photoreceptors of retina.

CONJUNCTIVA:

Mucous membrane that lines the eyelids and covers the exposed surface of the eye (the sclera).

CONJUNCTIVAL FORNIX:

The transition area between palpebral and bulbar conjunctiva.

CONJUNCTIVAL SAC:

Space limited by conjunctival and corneal surface.

CONJUNCTIVITIS:

Inflammation of the conjunctiva, usually associated with conjunctival hyperemia and discharge.

CORNEA:

Transparent structure forming the anterior part of the fibrous tunic of the eye.

CORNEAL DYSTROPHY:

Corneal disorder related to unknown pathogenesis, possibly related to heredity.

CYCLOPIA:

Developmental abnormality characterised by a single orbital fossa.

DEGENERATION:

Manifestation of cellular damage prior to necrosis.

DEMYELINATION:

Loss of myelin sheath around nerve fibres.

DESCEMET'S MEMBRANE:

Limiting membrane on the inner surface of the cornea.

DIPLOPIA:

The perception of a double image from a single object.

ECTROPION:

Outward turning of the margin of the eyelid thereby exposing further areas of the conjunctiva.

ELECTROMYOGRAPHIC:

Appertaining to the electrical responses of muscle.

ELECTRORETINOGRAM:

Measurements of electrical activity of the retina.

ELECTRORETINOGRAPHIC:

Appertaining to the electrical responses of the retina.

EMMETROPIA:

Normal refractive power.

ENOPTHALMOS:

Backwards displacement of the eyeball into the orbit.

ENTROPION:

Inward turning of the margin of the eyelid towards the eyeball.

EROSION:

Superficial damage to the epithelial surface.

EXOPHTHALMOS:

Abnormal protrusion of the eyeball.

EXTERNAL OPHTHALMOPLEGIA:

Paralysis of the external eye muscles.

FLUORESCEIN:

Fluorescent ophthalmic stain used to identify abnormalities.

FOLLICULAR CONJUNCTIVITIS:

Conjunctival inflammation associated with hyperplasia of conjunctiva- associated lymphoid tissue.

FOVEA CENTRALIS:

Small pit in the centre of the retina (only in primates).

FUNDOSCOPIC:

Pertaining to the fundus (see below).

FUNDUS:

Posterior part of eye visible through an ophthalmoscope.

GANGLION CELL LAYER:

Part of the retina cf Figure 2.

GLAND OF NICTITATING MEMBRANE:

Major ocular gland associated with the third eyelid.

GLAUCOMA:

Pathological condition associated with increased intraocular pressure.

GLIOSIS:

Proliferation of glial cells and fibrils in neural tissue analogous to scar formation.

GOBLET CELLS:

Mucus-secreting epithelial cells.

HARDERIAN GLAND:

Major ocular gland (absent in primates and carnivores).

HIPPUS:

Rhythmic contraction and dilation of the pupil independent of light intensity.

HORNER'S SYNDROME:

Sinking in of the eyeball, ptosis of the upper eyelid, slight elevation of the lower eye lid, constriction of the pupil.

HYPEROPIA:

Optical condition in which parallel rays of light come to focus virtually behind the retina ("long-sightedness")

INNER NUCLEAR LAYER:

Part of the retina, cf Figure 2.

INNER PLEXIFORM LAYER:

Part of the retina, cf Figure 2.

INNER SEGMENT:

Part of photoreceptor cells containing concentrated organelles.

INTERNAL LIMITING MEMBRANE:

Part of retina, cf Figure 2.

IRIDOCORNEAL ANGLE:

Located between iris and the cornea; the primary site of outflow of aqueous humour.

IRIS:

Diaphragm of the eye, responds to light intensity.

KERATITIS:

Inflammation of the cornea.

KERATOCONJUNCTIVITIS SICCA:

Inflammation of the cornea and the conjunctiva associated with lacrimal malfunction; "dry eye".

LACRIMAL:

Relating to the tears.

LAMINA CRIBROSA:

Site in the sclera where nerve fibres leave the eye.

LATERAL RECTUS MUSCLE:

Extrinsic ocular muscle which can move the eye in a lateral direction.

LENS:

Structure which focuses light rays on to retina.

MACULA LUTEA:

Retinal area containing a high concentration of cones.

MACULOPATHY:

Disease of the macula.

MEIBOMIAN GLAND:

Modified sebaceous glands of the eyelids.

MICROPHAKIA:

Developmental defect characterised by an abnormally small lens.

MICROPHTHALMIA:

Abnormal smallness of the eyes.

MIOSIS:

Constriction of the pupil.

MÜLLER'S MUSCLE:

Adrenergically innervated smooth muscle in the eyelid.

MYELINOPATHY:

Disease of the myelin sheath.

MYOPATHY:

Disease of muscle.

MYOPIA:

Optical condition in which parallel rays of light come to focus in front of retina ('short-sightedness').

MYOSITIS:

Inflammation of muscle.

NECROSIS:

Cell and tissue death.

NEOVASCULARIZATION:

Infiltration of tissue by new blood vessels.

NERVE FIBRE LAYER:

Part of the retina of Figure 2.

NEUROMUSCULAR ENDPLATE:

Motor endplate of nerve in contact with striated muscle to enable transmission of nerve impulses to muscle.

NEUROPATHY:

Lesion of neural tissue.

NEURAL RETINA:

All layers of the retina apart from pigment epithelium.

NICTITATING MEMBRANE:

Third eyelid, for example in birds, operates at right angles to the upper and lower eyelids.

NYSTAGMUS:

Involuntary rapid movement of the eyeball.

OCULAR IRRITATIVE RESPONSE:

Complex reaction consisting of an anterior uveal hyperemia, miosis, breakdown of the blood-aqueous barrier, a change in intraocular pressure and uveitis.

OCULAR TOXICITY:

The manifestation of abnormalities of the visual system due to exposure to xenobiotics.

OEDEMA:

Abnormal accumulation of extra-or intracellular fluid.

OPHTHALMOLOGY:

Clinical examination of the eye.

OPHTHALMOSCOPY:

Clinical examination of the eye fundus.

OPIDN:

Organophosphorus-induced delayed neuropathy.

OPTIC ATROPHY:

Atrophy of the optic nerve.

OPTIC DISC:

Ophthalmoscopically visible portion of the optic nerve in the globe.

OPTIC FASCICLE:

Anterior portion of the optic nerve, between the eyeball and the optic chiasm.

OPTIC NERVE APLASIA:

Failure of the optic nerve to develop correctly.

OPTIC NERVE PAPILLA:

"Blind spot": cf Figure 1, the point of exit of the optic nerve out of the eyeball.

OPTIC TRACT:

Optic nerve behind the optic chiasm, leading to the brain.

OUTER NUCLEAR LAYER:

Part of the retina, cf Figure 2.

OUTER PLEXIFORM LAYER:

Part of the retina, cf Figure 2.

OUTER SEGMENT:

Part of photoreceptors containing stacks of membrane-bound discs.

PACHYMETRY:

Measurement of corneal thickness.

PALPEBRAL CONJUNCTIVA:

Mucous membrane covering inner surface of eyelids.

PALPEBRAL TREMOR:

Tremor appertaining to the eyelids.

PAPILLOEDEMA:

Oedema of the optic disc.

PECTEN:

Triangular pleated membrane in the eye of birds extending forward from the optic disc, which it covers, for a variable distance into the vitreous body.

PERFORATION:

An opening or hole produced in a tissue or structure by a pathologic process.

PERIFOVEAL:

Around the fovea (the avascular region in the centre of the macula containing only cone photoreceptors).

PHOTOPIC:

Appertaining to the cone photoreceptors which are active mainly in the light-adapted state.

PHOTORECEPTOR:

Light sensitive cell, cf. Figure 2.

POSTERIOR CHAMBER:

cf. Figure 1.

PIGMENTARY RETINOPATHY:

Disease of the retina with changes in pigmentation.

PTOSIS:

Drooping of the upper eyelid.

PUPIL:

Point of entry of light into the eye in the middle of the iris.

PUPILLARY MEMBRANE:

Anomaly of iris characterised by persistence of foetal pupillary membrane.

PUPILLARY REFLEX:

Reaction of pupil to light stimulation.

RETINA:

Light-receptive innermost layer of the eyeball wall, cf Figure 2.

RETINAL LIPIDOSIS:

Deposition of lamellated bodies within the retina.

RETINAL NEURONOPATHY:

Degeneration of neurones associated with the retina.

RETINAL PIGMENT EPITHELIUM:

Pigmented outer retinal layer, except in albinos.

RHABDOMYONECROSIS:

Death of striated muscle.

SACCADIC EYE MOVEMENT:

Series of involuntary, abrupt, rapid, small movements of both eyes simultaneously in changing the point of fixation.

SAKU DISEASE:

Spectrum of ocular changes in man, reported in Japan and allegedly attributed to exposure to organophosphorus pesticides.

SCLERA:

Tough white outer coat of the eyeball, cf Figure 1.

SCOTOMA:

Area of depressed vision within the visual field.

SCOTOPIC:

Appertaining to the rod photoreceptors which are mainly active in the dark.

STRABISMUS:

Deviation of the orientation of the eye from the norm, a squint.

TAPETUM LUCIDUM:

Layer of specialised cells in choroid which reflect light (absent in man, most non-human primates and rodents).

TUBULIN:

Principal protein of the fibres comprising the mitotic spindle.

ULCER:

Damage to the epithelial surface with progression into the deeper tissues.

UVEA:

Middle coat of the eye, including the iris, the ciliary body, the choroid and the tapetum lucidum.

UVEITIS:

Inflammation of the iris and ciliary body and choroid.

VASOCONSTRICTION:

Constriction of blood vessels.

VITREORETINAL DYSPLASIA:

Abnormal development of the vitreous body and retina.

VITREOUS BODY:

Hydrogel filling the interior cavity of the eye, cf Figure 1.

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